The role of dietary patterns, inflammatory status and gut microbiome in bone health maintenance of postmenopausal women – A cross-sectional study

A thesis presented in fulfilment of the requirements for the degree of

Doctor of Philosophy

in

Nutritional Science

School of Health Sciences

Massey University,

Palmerston North

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2020

To

my loving husband

Peter

and our blessings

Dafidi III

&

Maria II

Abstract

The incidence of postmenopausal osteoporosis (a disease in which bones become weak and brittle) is increasing in parallel with the ageing of the global population. Postmenopausal osteoporosis is characterized by increased low grade inflammation that contributes to low bone mass and degradation of bone mineral density (BMD) resulting in postmenopausal bone loss. Elevated levels of pro-inflammatory cytokines such as IL-6, TNF-α, IL-1β and RANKL are produced by activated T-cells inducing osteoclast formation and activity during senescence.

The objective of the "Bugs'n'Bones" clinical study was to assess the relationship between dietary patterns, inflammatory status, gut microbiota and bone health status in New Zealand postmenopausal women. We hypothesised that lack of physical activity, increased intake of poor diets, low in fibre and nutrients, and high in fat, salt and/or sugar will increase chronic inflammation and reduce BMD.

The results of this human study indicated that alongside improving physical activity status with increase in lean body mass, a nutrient pattern with high loadings of B-vitamins, calcium and phosphorus was related to an increase in BMD. In addition, dietary pattern with a high factor loading of milks and milk-rich beverages was associated with a high BMD and T-score. The results of this study also indicated that a high circulatory level of inflammatory cytokines was associated with lower BMD in the postmenopausal women. The results of the gut microbiota analyses showed that microbial composition diversity (alpha diversity by Shannon index) was significantly lower amongst the osteopenic/osteoporotic groups than their healthy counterparts. The interferon gamma receptor 1 orthology group was significantly higher in abundance for the OP (osteopenic/osteoporotics) than the H (healthy) groups based on the hip and femoral neck osteoporosis status.

Future longitudinal and intervention studies which aim to modulate the gut microbiota via dietary change is warranted. It is also important to conduct these interventions with prebiotics, probiotics and synbiotics in animal and human models.

Acknowledgements

First and foremost, I am grateful to the Almighty Lord God for the gift of life and for enabling me to accomplish this milestone. 'For You, O Lord, will bless the righteous with favour; You will surround him as a shield' – Psalm 5:12.

I had like to thank my extraordinary supervisor, Prof. Marlena Kruger for your amazing support and for always being gracious and approachable. Thank you for single-handedly sourcing the funding for this work. May God's goodness, mercy and love continue to overshadow you.

I would also like to thank my affable co-supervisors, Prof. Nicole Roy (for partly funding the research) and Prof. Jane Coad for their commitment and mentorship. Thank you and may God richly bless you.

I am grateful for the contributions and advice from Prof. Michelle McConnell, Dr. Elizabeth Rettedal, Dr. Linda Schollum, Dr. Barbara Kuhn-Sherlock and Dr. Louise Brough.

Indeed, I am highly indebted to Riddet Institute for awarding me a scholarship for my PhD studies. I would also like to acknowledge Palmerston North Medical Research Foundation, Fonterra Cooperative Group Ltd and Allen Foundation, USA for funding this research as well as the School of Food and Advanced Technology, Massey University for financial and resource support throughout my PhD. I thank AgResearch Limited for providing resources for the gut microbiota analyses and thanks to Massey Genomics Services (MGS) and Otago Genomics and Bioinformatics Facility (OGBF) for their help with DNA prep and sequencing respectively.

The technical expertise and assistance from Annie Broomfield for conducting the DXA for the ladies, Gabby Plimmer for the ELISA tests, Shampa De for the DNA extractions (Massey University), Sonya Mros and Katie Young for the cytokine analyses (Otago University) are greatly appreciated. Thank you, Mrs. Anna Willoughby, for your help with blood collection. The knowledge, expertise and help from the librarians; Chris Good, Anne Ram, Lorraine Tremain, Barbara Rainier and Jeff Phillips is greatly treasured.

The prayers and words of encouragement from friends (RoAnn Smith, Sewuese Okubanjo) and family (Prof. Isaac and Dr. Doris Adeyinka, Dr. Raphael and Mrs. Evelyn Folorunsho, The Oyelere's, The Ilesanmi's family) are much appreciated. I am grateful to Mr. Samuel Oyelere and family for their help with my family's travel tickets while I study.

Thanks to my Riddet building office colleagues Blue, Saima and Umani for a great working environment and all Riddet Complex level 3 tea room group (Dr. Fran Wolber, Louise Shaw, Dr. Sarah Bond, Corrin Hulls, Nikhi Vijay and Giovanna).

My gratitude also goes to the support from my AgResearch office colleagues from Allan Johns' building room F17. My thank you goes out to Mrs. Nicky Rees for her counselling words.

I would also like to say a big thank you to all the participants of the “Bugs’n’Bones” clinical study.
Finally, and definitely not the least, I had like to thank my dear husband Prof. Peter B. Oyelere and our beautiful children Dafidi III Oyelere and Maria II Oyelere who constantly supported and encouraged me throughout my PhD journey. Thank you all.

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List of Abbreviations

3-DDD	3-day diet diary
AEE	Activity energy expenditure
ALP	Alkaline phosphatase
ANOVA	Analysis of variance
ANZCTR	Australia New Zealand clinical trials registry
ATP	Adenosine tri-phosphate
BALP	Bone-specific alkaline phosphatase
BMC	Bone mineral content
BMD	Bone mineral density
BMI	Body mass index
BMU	Basic multicellular unit
BSP	Bone sialoprotein
CI	Confidence interval (95%)
CRP	C-reactive protein
CTX-1	C-terminal telopeptide
DNA	Deoxy-ribonucleic acid
DPD	Deoxypyridinoline
DXA	Dual-energy X-ray absorptiometry
ECLIA	Electrochemiluminescence immunoassay
ELISA	Enzyme-linked immunosorbent assay
FDR	False discovery rate
FFQ	Food frequency questionnaire
FM	Fat mass
FN	Femoral neck
GOS	Galacto-oligosaccharide
H	Healthy group
IFN	Interferon
IGF-1	Insulin growth factor-1
IL	Interleukin
KO	Kegg orthology
LM	Lean mass
LS	Lumbar spine
MET	Metabolic equivalent
MCP-1	Monocyte chemoattractant protein
MGS	Massey genomics service

MMP	Matrix metalloproteinase
MUFA	Mono-unsaturated fatty acid
NMDS	Non-metric multidimensional scaling
NTX	N-terminal telopeptides of type 1 collagen
NZPAQ-SF	New Zealand physical activity questionnaire short form
OC	Osteocalcin
OGBF	Otago genomics & bioinformatics facility
OP	Osteopenic/Osteporotic group
OTU	Operational taxonomic unit
P1CP	Pro-collagen type 1 C-terminal propeptide
P1NP	Pro-collagen type 1 N-terminal propeptide
PAEE	Physical activity energy expenditure
PCA	Principal component analysis
PCoA	Principal coordinate analysis
PUFA	Poly-unsaturated fatty acid
PYD	Pyridinoline
QALY	Quality-adjusted life year
QUS	Quantitative ultrasound
RANKL	Receptor activator of nuclear factor kappa-B ligand
REE	Resting energy expenditure
RPB 11	DNA-directed RNA polymerase 11
RNA	Ribonucleic acid
SCFA	Short chain fatty acid
SPSS	Statistical Package for the Social Sciences
TEF	Thermic effect of food
TGF	Transforming growth factor
TRACP	Tartrate-resistant acid phosphatase
TNF	Tumour necrosis factor
WC	Waist circumference
WH	Waist to hip ratio
WHO	World Health Organization

List of published papers

1. Ilesanmi-Oyelere, B.L.; Roy, N.C.; Coad, J.; Kruger, M.C. Associations between Self-Reported Physical Activity, Heel Ultrasound Parameters and Bone Health Measures in Post-Menopausal Women. Int. J. Environ. Res. Public Health 2019, 16, 3177. https://doi.org/10.3390/ijerph16173177
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List of presentations

Parts of this PhD project have been presented at the following forums:

1. Centre for Metabolic Health Research Annual Symposium, College of Health Sciences, Massey University (June 8, 2017) ~ Oral presentation
2. The 7th International Symposium on "Delivery of Functionality in Complex Food Systems", Auckland (November 5 – 8, 2017) ~ Oral presentation
3. Centre for Metabolic Health Research Annual Symposium, College of Health Sciences, Massey University (June 8, 2018) ~ Oral presentation
4. Annual Meeting of the Nutrition Society of New Zealand on "Finding the Balance", Auckland (November 27 -30, 2018) ~ Poster presentation
5. The 5th International Conference on Food Structures, Digestion and Health on "Working With and Like Nature", Rotorua (September 30 – October 3, 2019) ~ Poster presentation
6. The 13th European Nutrition Conference (FENS 2019) on "Malnutrition in an Obese World: European Perspectives", Dublin, Ireland (October 15 – 18, 2019) ~ ePoster presentation
7. Annual Meeting of the Nutrition Society of New Zealand on "Beyond Nutrition", Napier (November 27 -29, 2019) ~ Oral presentation

Chapter 1 Introduction

Osteoporosis is known as 'porous bone' which is derived from the Greek words 'osteoun' (bone) and 'poros' (pore) and is characterised by low bone mass and microarchitectural deterioration of bone tissues consequently increasing bone fragility and breakage [1]. The porosity of osteoporotic bone generally results from an imbalance in the bone remodelling process whereby the rate of resorption by osteoclasts is greater than formation by osteoblasts [2]. This leads to a reduction in the density and quality of bone.

The clinical diagnostic criteria and intervention threshold widely acceptable is defined as a bone mineral density (BMD) in older men and postmenopausal women more than or equal to 2.5 standard deviations (T score of $\leq$ -2.5) at the lumbar spine, femur neck or total hip bone below the mean value of the young reference [3].

The categories for diagnosis are:

- Normal (T-score -1.0 and above)
- Low bone mass, referred to as osteopenia (T-score between -1.0 and -2.5)
- Osteoporosis (T-score -2.5 and below)
- Severe osteoporosis (T-score -2.5 and below with history of fracture)

The T-score is calculated from the BMD.
[T-score = (BMD – reference BMD) / SD]

The incidence of postmenopausal osteoporosis is increasing with the ageing global population. The osteoporosis epidemic is a major public health concern especially among postmenopausal women with a lifetime risk of wrist, hip or vertebral fracture estimated to be >40% for women in developed countries [4]. As a global epidemic situation, the International Osteoporosis Foundation suggests that one in three women and one in five men over 50 years of age will experience an osteoporotic fracture [5]. Likewise, the World Health Organisation (WHO) reported that the number of osteoporotic fractures is set to increase by more than 3-fold over the next 50 years as a result of the ageing population [6].

The vast burden of osteoporosis constitute increased morbidity and mortality [7], loss of quality-adjusted life years (QALYs) [8], and a continuous rise in the cost of healthcare services such as clinics, nursing homes and hospitals [9]. It is a growing global health concern with the lifetime risk of sustaining any fracture at ~50% for women and ~20% for men in individuals over 50 years living in developed countries [10].

In New Zealand, the incidence rate of osteoporotic fractures has been estimated to be >84,000, with 60% occurring in women. QALYs are significantly reduced due to osteoporotic fractures. In 2007, results show that nearly 2,100 QALYs are lost per year; 935 in females [8]. In 2011, osteoporosis related QALYs lost was found to be 11,249 with a projection of 13,205 and 15,176 for 2013 and 2020 respectively due to fractures. The results also suggest there are more QALYs lost in women (6,028) more than men (5,221) [11].

The disease constitutes an economic burden particularly due to the high cost of treatment. The burden of osteoporosis also relates to the use of health services and cost of care, with a total cost estimated to be over $1.15 billion per annum and an increase of over 30% expected between 2007-2020 [8]. Over $300 million per annum is estimated to be spent on treating fractures while total costs are estimated at $1.15 billion per annum in health costs, posing a heavy burden on healthcare service providers in New Zealand [9].

With an ageing population and an increased awareness of the rising health cost of osteoporotic fractures; the focus of osteoporosis research is changing from treatment to prevention. Bone loss associated with ageing involves a multifaceted interaction with genetics, body/frame size and family history playing a major role in bone health. However, this can be modified by nutrition (especially calcium, vitamin D, vitamin A, vitamin K, protein, phosphorus and omega-3 fatty acids intake). Physical activity, lean body mass, hormone and vitamin D levels, lifestyle factors (excessive alcohol intake and cigarette smoking) and some medications (such as corticosteroids and aromatase, selective serotonin reuptake and proton pump inhibitors) also play a role in the modification of bone health [2].

Bone health issues pose a lifetime concern. Nevertheless, available evidence indicates that optimal nutrition could significantly alleviate or reduce the risks and incidence of bone-related diseases [12]. Nutrition can be both a powerful and economical tool in tackling the growing public health problems of chronic diseases such as osteoporosis, arthritis, diabetes, cardiovascular diseases, hypertension, cancer etc. This is particularly true because of our inability to modify our genetic make-up, environment or ethno-demography. However, we can focus on diet modification.

Different life stages have varied nutrient utilization capacity, for example, efficiency of calcium absorption would differ through infancy to the elderly. Next question is how much of these nutrients is needed, especially since nutrients are interdependent for bioavailability [13].

The impact of many nutrients and food components we consume on bone health can be profound. Just like the harmony in a symphony orchestra, all nutrients are required at the recommended amount to improve bone health due to their interdependent status. Western diets are made up of nutrients/food components that can be either detrimental or beneficial depending on the dietary exposure level [12].

Several factors are known to alter BMD, of which dietary patterns and change in lifestyle can be easily managed. Research has been mainly reductionist in nature regarding the relationship between diet and BMD, focussing generally on a single nutrient or food rather than the need to consider a combination of nutrients such as the dietary pattern of individuals [14]. It has therefore been suggested that investigating a combination of foods as described by dietary patterns owing to the differences in food preferences as a result of social, cultural, economic, health, environmental and lifestyle factors might be more appropriate to study [14].

Another factor that has been proven to alter both the innate and adaptive immune responses is the gut microbiota [15]. Diet (nutritional intervention) is known to play a major role in driving the composition and metabolism of the gut microbiota which can rapidly and reproducibly alter the gut microbiome subsequently affecting the individual's health [16]. The common feature of microorganisms used as commensal probiotics is their ability to protect against inflammation. This is due to the detrimental effects of high levels of inflammatory cytokines on bone status [17].

The composition of the gut microbiome, however, has been known to be shaped by the individual's genetics, birth method, infant feeding patterns, antibiotic usage, sanitary living conditions as well as long term dietary habits. The effect of the gut microbiome has also been indicated in the pathology of several intestinal inflammatory diseases, cardio-metabolic disorders as well as in the development of colorectal, gastric, and prostate cancers [18].

Recent studies in rats and mice suggest that the gut microbiome modulates the immune status [19], calcium absorption and molecular control of bone resorption [20-22]. Although research studies have been conducted in animals, clinical trials of human studies are few and no research in the New Zealand has looked into the relationship between diet, gut microbiome and postmenopausal osteoporosis. Moreover, the human microbiota is different from that of rodents which is the reason why many studies have faced limited success in their attempt to 'humanise' the murine microbiota [23]. The overarching aim was to investigate the relationship between diet, inflammation, gut microbiota and bone health in postmenopausal women. To achieve this, the research seeks to answer the following questions.

1.1 Research questions

1. What are the nutritional/lifestyle factors that influence the bone density status?
2. How does specific nutrients and nutrient patterns affect the bone density?
3. What are the effects of New Zealand food-based dietary patterns on the bone density status?
4. What are the relationships between inflammatory status, BMD and bone biomarkers amongst postmenopausal women?
5. How does the gut microbiota affect the bone density?

1.2 Specific objectives

1. To assess participants' body composition and physical activity in relation to their bone density status.
2. To determine the relationship between bone-related nutrients, nutrient patterns (identified using 3-day diet diary) and BMD.
3. To evaluate the relationship between participants' dietary patterns (identified using the food frequency questionnaires) and BMD/T-scores.
4. To assess the relationship between participants' inflammation status, BMD and bone biomarkers.
5. To identify the association between gut microbiota and bone density status among the individuals.

1.3 Hypotheses

1. Individuals with higher lean body mass and vigorous physical activity will have a better bone density profile due to the effect of weight, mechanical actions and force on the bone.
2. The regular consumption of nutrient-dense foods will have a desirable effect on the bone density status.
3. Dietary patterns with a high loading of healthy foods will have positive correlations with bone health indicators such as BMD status.
4. Individuals with low BMD will have greater pro-inflammatory profile than those with higher BMD.
5. Non-osteoporotic individuals will harbour differing gut microbiota compared to the osteoporotic groups.

1.4 Thesis structure

This introduction chapter is followed by the literature review (Chapter 2) that provides an overview of the available studies on the association between diet and bone health. The literature review explores the influence of gut microbiota and inflammatory cytokines on bone health. Chapter 3 demonstrates the impact of self-reported moderate-vigorous physical activity on bone health status. Chapter 4 provides the relationship between body compositions (for example, lean body mass and fat mass) and BMD. In Chapter 5, the relationship between selected nutrients and nutrient patterns generated from the 3-day diet diary (3-DDD) data and bone parameters were analysed and reported. Chapter 6 provides the association between dietary patterns derived from the food frequency questionnaires (FFQ) and spine, hip and FN BMD/T-scores. In Chapter 7, analyses of the association between inflammatory cytokines, BMD and bone biomarkers were reported and illustrated. Chapter 8 discusses the relationship between the gut microbiota and bone health parameters. Finally, the discussion, conclusion and areas of future research are outlined in Chapter 9.

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Chapter 2 Literature Review

2.1 Introduction

In order to investigate the pathogenesis and aetiology of osteoporosis, it is important to inquire and consider the modifiable and non-modifiable risk factors that affect bone health in human especially postmenopausal women. In addition, to understand the contributions of lifestyle, diet, gut microbiota and inflammation to osteoporosis in older age, it is of importance to review previous studies showing relationship, association or effect of these risk factors on bone health from childhood to adulthood.

In this review, literature on bones, bone turnover markers, dietary patterns and gut microbiome, inflammatory cytokines and their impact on bone density and status were researched. In-depth analysis of other factors such as diet, gut microbiome, ethnicity and lifestyle issues contributing to bone health were also reviewed.

2.2 Bone

2.2.1 Types of bones

The human skeleton is made up of five types of bones.

The long bones – they are wide, and they support weight to enable movement of the body. Examples of long bones are the lower limbs which include the tibia, femur, fibula, phalanges and metatarsals and the upper limbs, that is, the humerus, ulna, radius, phalanges and metatarsals [1, 2].

The flat bones – mainly protect the internal organs and it also provides attachment for the muscles. Examples of flat bones include the skull (occipital, frontal, parietal, lacrimal and vomer), the thoracic cavity (ribs and sternum) as well as the pelvis (pubis, ischium and illium) [3].

The short bones – normally cube-shaped, they provide stability and some movement. Examples of this include the carpals in the wrist, that is, the lunate, scaphoid, pisiform, trapezium, trapezoid, triquetral, hamate and capitate, and the tarsals in the ankles, that is, calcaneous, cuboid, talus, navicular, and lateral, intermediate and medial cuneiform [4].

The irregular bones – possess varying complex shapes that gives the name and they protect internal organs and the spinal cord. Examples include the vertebral column and pelvis [2, 4].

Sesamoid bones – are incorporated into the tendons for reinforcement, for protection and to prevent stress due to the wear and tear of the joints. These bones are commonly found in the tendons of the knees, hands and feet for example; the knee cap also known as patella [4, 5].

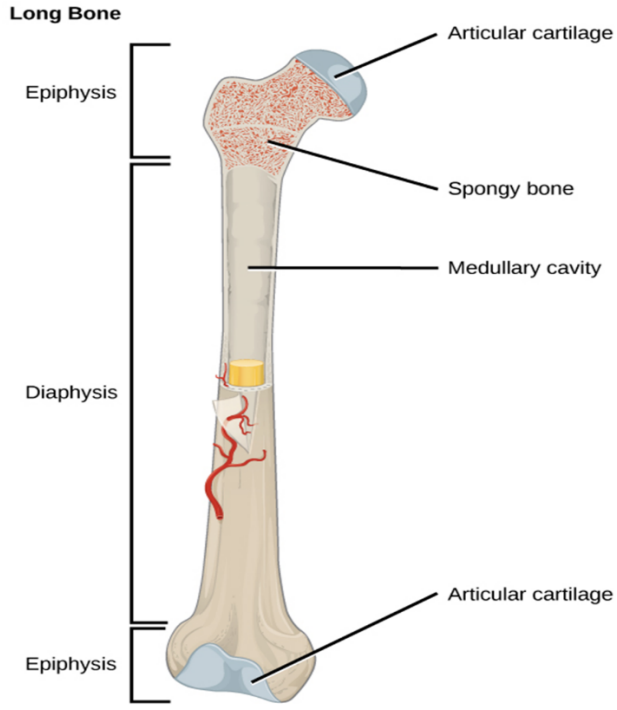

Figure 2-1 The anatomy of a long bone

Figure 2-1 shows the anatomy of a long bone. Reproduced with permission from Openstax ™ [2].

2.2.2 Bone structure and cells

Bone is a complex living tissue that undergoes a continuous renewal in order to maintain the mechanical competence of the bone matrix. Aside from its role in providing mechanical support to the body, it aids movement and protects soft tissues and vital organs. There are two types of tissue present in a mature bone: the thick and compact (cortical) bone which forms the top layer of most bones (for example long bones) comprising 80% of the adult skeleton and the trabeculae (cancellous) bone which is less dense and spongy-like with many small openings consisting of 20% by mass [6]. Bones are responsible for storage and release of vital minerals such as calcium, phosphate, growth factors and other minerals including magnesium and fluoride. The skeleton has been compared metaphorically to a giant ion exchange column [7] involving the deposition and withdrawal of minerals dependent on cellular and serum regulatory mechanisms. A constant supply of bone-building minerals (calcium, phosphorus, magnesium, boron, zinc, copper and manganese) is required as well as vitamin D and K in order to maintain reserves in the skeletal system [8] for mineral homeostasis. The skeleton is also responsible for fat-triglyceride storage (yellow marrow present in long bones) as well as blood cell production (red marrow) [6].

The chemical component of the bone comprises of about 25% water, approximately 25% organic constituent which is mainly type I collagen (a fibrous protein that provides flexibility), proteoglycans and glycoproteins. The remaining bone chemistry include approximately 50% inorganic constituent, calcium phosphate ($CaPO_4$) and calcium carbonate ($CaCO_3$); mineral salts that provide the rigid strength and density. Hydroxyapatite ($Ca_5(PO_4)_3(OH)$) is a major essential part of the bone. Bone has the ability to grow and synthesise new bones from bone cells. There are four types of bone cells with a central role in bone metabolism: chondrocytes (cartilage forming), osteoblasts (bone forming), osteocytes (maintenance/regulation of both osteoblasts and osteoclasts) and the osteoclasts (bone resorbing) [7].

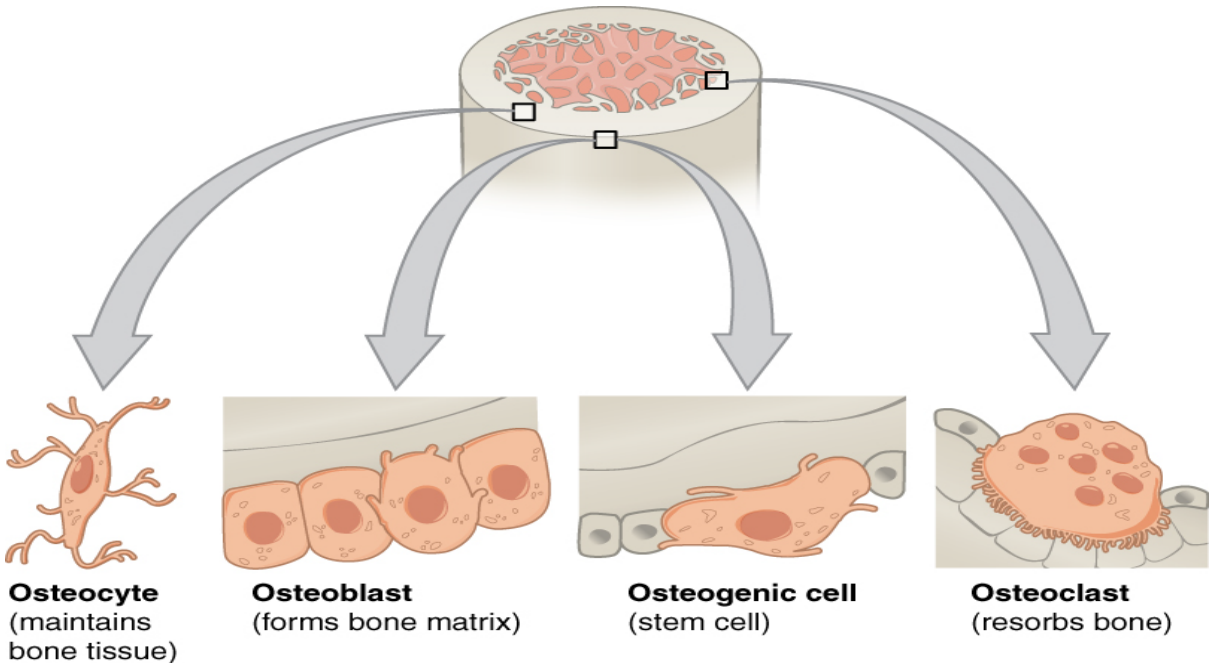

Figure 2-2 Bone cell types

Figure 2-2 depicts the structure of four bone cell types. Osteocytes known as the most abundant cell type in the bone and lies within the matrix of the fully formed bone. Osteoblasts are cells with a nucleus that synthesis and mineralize bone. Osteogenic cells are the bone cells that divide and develop into osteoblasts. Osteoclasts are large multinucleated cells with a ruffled membrane while the other cell types are mononucleated. Reproduced with permission from Openstax™ [2].

2.2.2.1 Osteoprogenitors

Chondrocytes are derived from the mesenchymal stem cells also known as osteogenic cells. They contain an oval or a round nucleus and are pluripotent in nature. Chondrocytes function as important cells for growth. The proliferation of chondrocytes produces type II collagen-rich template for the cartilage and they secrete type X collagen followed by apoptosis; meanwhile the resultant mineralised cartilage forms the basis for bone formation [9]. They become hypertrophic by terminal differentiation during endochondrial ossification and may transdifferentiate to osteoblasts under the influence of growth factors [10].

2.2.2.2 Osteoblasts

Osteoblasts originate from mesenchymal stem cells (MSC) and sometimes cells of bone lining and chondrocytes. Osteoblasts are required for production of type 1 collagen-rich bone matrix and mineralisation of the tissues. The osteoblasts synthesise the extracellular matrix proteins osteocalcin and osteopontin to make up the osteoid. Osteoblasts also produce alkaline phosphatase (an enzyme responsible for forming hydroxyapatite; the mineral portion of bone). Once the osteoblasts have synthesised sufficient organic and inorganic portions of the bone matrix; they mature into osteocytes. Other osteoblasts may undergo apoptosis or revert back to lining cells to cover the surface of bone. Wingless type (Wnt) signalling pathway is also known to control osteoblastogenesis [10].

2.2.2.3 Osteocytes

Osteocytes are a subpopulation of long-lived osteoblasts embedded in the bone, which play a role in bone homeostasis. They occupy the lacunae ‘empty space’ part of the bone. The osteocyte branches allow for communication with other cells for bone maintenance. They provide a network of cellular structure and cell processes in the canaliculi of the matrix. The network of osteocytes enable detection of any microdamage to the bone as a result of mechanical demands by converting mechanical forces to biochemical signals [11]. Osteocytes are the most abundant mature bone cell types.

2.2.2.4 Osteoclasts

Osteoclasts are derived from haematopoietic stem cells and directly from monocytes and macrophage precursors which are multinucleated cells. The acidic environment of the ‘sealed zone’ around which the osteoclast is able to dissolve bone mineral matrix is produced by the podosomes which consist of an actin core made up of integrins and cytoskeletal proteins in the osteoclasts. Osteoclastogenesis is accomplished by the actions of proteolytic enzymes such as cathepsin K and tartrate resistant acid phosphatase (TRAP) on the bone collagenic matrix. They also contain carbonic anhydrase, used for production of H^+ ions secreted at the ruffled border.

The resulting osteoclastogenesis by osteoclasts may be induced or aggravated by immune cells as result of inflammation [9].

2.2.3 Bone remodelling

Bone as a dynamic entity is constantly renewed and the continual wear and tear requiring repair and maintenance, through the bone remodelling cycle. Bone remodelling comprises five sequential phases: activation, resorption, reversal, formation by matrix deposition and termination after mineralisation [12]. The bone remodelling cycle takes place within the basic multicellular unit (BMU), containing the osteoblasts, osteoclasts and the capillary blood supply [9]. Bone turnover balance is achieved and regulated by circulating hormones such as parathyroid hormone (PTH), calcitonin, gonadal steroids and vitamin D_3 and local mediators such as cytokines and growth factors [13, 14]. It is widely accepted to be affected by the degree of inflammation [15, 16] and an imbalance may result in reduction of bone mass (subsequently bone loss). The bone remodelling cycle is highly dependent on the interactions of two cell lineages namely the mesenchymal osteoblastic lineage and the hematopoietic osteoclastic lineage [17].

2.2.3.1 Activation phase

The first stage of bone remodelling is the activation. The signalling process begins due to the response of osteocytes to functional mechanical strain (mechanosensing), lack of mobility or damage to the resting bone cells [12]. Furthermore, this biological signalling can result from cytokines, oestrogen and growth factor actions or PTH (calcitropic hormone) in response to calcium homeostasis. Parathyroid receptors found on the osteoblasts fuse and results in the activation of protein kinases A and C and calcium intracellular signalling pathways [18]. The activation stage involves the interaction of the precursor cells of osteoclasts, that is, the osteocytes leading to differentiation, migration and fusion of the large multinucleated osteoclasts to result in osteoclast activation and ultimately bone resorption [17].

2.2.3.2 Resorption phase

Osteoblasts, in response to signals by osteocytes, endocrine activation and PTH-induced bone remodelling produce monocyte chemo-attractant protein-1 (MCP-1) *in-vivo*. The homing signals of MCP-1, a chemokine leads osteoclast precursors to the remodelling site. In addition, MCP-1 enhances RANKL-induced osteoclastogenesis *in vitro* [12, 13]. Expression of osteoclastogenic cytokines, CSF-1, RANKL and osteoprotegerin (OPG) are modified by the PTH while MCP-1 reduces the expression of OPG and increases CSF-1 and RANKL production thereby promoting osteoclast formation [12, 17, 18].

Osteoclasts secrete matrix metalloproteinases (MMPs) in response to mechanical, and endocrine remodelling signals [12]. The degradation of the unmineralised osteoid lining cells of the bone surface by MMPs exposes Arginine-Glycine-Aspartate (RGD) adhesion sites located within mineralised bone facilitating the attachment of osteoclasts. The cells attach to the mineralised bone surface subsequently initiating resorption through hydrogen ions and lysosomal enzyme secretion, especially cathepsin K which degrades the bone matrix including collagen at a low pH [12, 18].

The bone resorption process involves two parts; acid (H^+ Cl^-) secretion dissolves by disintegrating the inorganic matrix and cathepsin K then generates proteolysis [19]. Cavities produced as a result of osteoclastic resorption are called Howship resorption lacunae in the trabecular bone surface and cylindrical Haversian canals in the cortical bone surface [17] due to the differences in the structure and composition of their BMU. The resorption phase usually takes approximately two weeks [9].

2.2.3.3 Reversal phase

On the completion of bone removal by the osteoclasts, the reversal phase is initiated by mononuclear cells of macrophage lineage otherwise known as the reversal cells [10]. During this phase, bone resorption begins its transformation to formation. This phase is however still not well understood. However, in this phase it is known that the resorbed bone surface commences it preparation for bone matrix deposition coupled with many more signalling occurring between resorption and formation in order to prevent a net bone loss [10]. The duration of this phase takes about four to five weeks [9].

2.2.3.4 Formation phase

The formation stage involves the filling of the cavity, created during the resorption phase with several layers of osteoblasts from mesenchymal precursor cells and a deposition and accumulation of mineralised matrix. Insulin-like growth factors I and II plus TGF-β have been reported to be key factors for recruiting mesenchymal cells to the resorption site [12]. When early osteoblast precursors or mesenchymal stem cells move back to the resorption lacunae, differentiation takes place and organic matrix called osteoid (collagen) is secreted. Bone replacement molecules such as Ca^{2+} and PO_4^- are then generated. This process of formation takes approximately four months in normal individuals [9].

2.2.3.5 Termination phase

Following the mineralisation stage is the final termination phase. Mature osteoblasts thereby undergo apoptosis resulting in either entombment into mineralised matrix or are reverted back into the bone-lining cells from osteocytes by differentiation [12] ready for another cycle of bone remodeling in its resting state. Osteocytes are known to be instrumental in the Wnt signaling pathway that signifies the end of bone remodeling by producing osteogenesis antagonists such as proinflammatory interleukins and TNF-α [20].

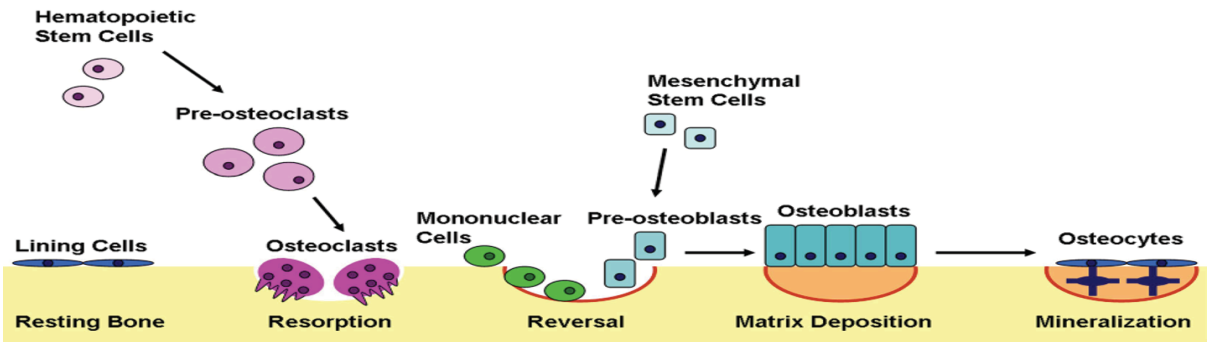

Figure 2-3 Bone remodelling

Figure 2.3 shows the bone remodelling process with the sequential phases. Reproduced with permission from Kapinas and Delany 2011 [20].

2.2.4 Bone turnover biomarkers

Parfitt 2002 and 2004 explained the definition and process of bone turnover excellently as "the total volume of bone resorbed or formed over a period of time which can be expressed as percent or year and quantified by measuring the bone biomarkers" [21, 22]. Markers of bone turnover found in blood (serum or plasma) are used by clinicians and researchers to determine the effects of treatment and BMD changes in osteoporosis research. The analytes in serum or plasma and urine are used for research purposes and unsuitable for final diagnosis of osteoporosis. Automated, manual immunoassay and multiplex microarrays are used for analysis of bone turnover markers. They are prone to circadian, analytical and inter-person variations therefore the use of automated standardised assays are recommended [23]. In addition, to compensate for the circadian variation, blood tests are scheduled for the same time of the day and usually after fasting overnight. The International Osteoporosis Foundation has also proposed serum CTX-1 (sCTX) and serum PINP (sPINP) for use as reference markers of bone resorption and formation respectively for fracture risk and therapy monitoring in clinical settings [24].

2.2.4.1 Bone resorption markers

Bone resorption markers are the by-products of the bone resorption phase during the osteoclastic activity of the bone remodelling process. The N- and C-terminal telopeptides ends of mature collagen released during bone resorption can be detected in the circulation [23, 25]. Other bone resorption markers include collage degradation by-products such as pyridinoline (PYD) and deoxypyridinoline (DPD) as shown in Table 2-1. Osteoclastic enzymes produced as a result of bone resorption include Tartrate-resistant acid phosphatase (TRACP), cathepsin K and matrix metalloproteinases (MMPs) [25].

2.2.4.2 Bone formation markers

Osteocalcin is secreted by mature osteoblasts. It is a 49-amino acid, calcium-binding peptide [26]. Osteocalcin is the most abundant non-collagen protein in the bone [26]. The type I collagens are known to be part of the bone matrix. Osteoblasts release its precursor, type I procollagen which undergoes proteolytic cleavage resulting in amino terminal and carboxy-terminal propeptides of type I collagen (PINP, PICP) [23]. The rate of bone formation concentrations are thought to be reflected in the rate of the circulatory levels of PINP and PICP in the blood [23, 25]. The osteoblast enzymes are ALP and BALP.

2.2.4.3 Clinical utility of bone biochemical markers

Bone biochemical markers are used in predicting bone loss, BMD and risk of fractures [27]. It is also important for monitoring osteoporosis treatment by anti-resorptive agents such as bisphosphonates and denosumab. Based on clinical studies, high marker levels predict higher rate of bone loss and increased non-traumatic risk of fracture independent of confounding factors such as age or sex [28]. Elevated levels of bone biochemical markers (serum BSAP and PTH) are used to detect kidney disease in nephrology, oncology and rheumatological conditions [29]. High bone turnover resulting in elevated bone markers are also used in the diagnosis and monitoring of Paget's disease of the bone [28]

Table 2-1 Bone turnover markers

Marker	Full name	Assay	Clinical source	Remark
Resorption				
PYD	Pyridinoline	Automated Manual	Urine	Collagen degradation by-products
DPD	Deoxypyridinoline	Automated Manual	Urine	Collagen degradation by-products
NTX	Telopeptides of type I collagen: N-terminal	Automated Manual	Serum, plasma or urine	Collagen degradation by-products
CTX-1	Telopeptides of type I collagen: C-terminal	Automated Manual	Serum, plasma or urine	Collagen degradation by-products
BSP	Bone sialoprotein	Automated Manual	Serum	Non-collagenous proteins
TRACP	Tartrate-resistant acid phosphatase	Manual	Serum	Osteoclastic enzymes
Cathepsin K	Cathepsin K	Manual	Serum	Osteoclastic enzymes
MMPs	Matrix Metalloproteinases	Manual	Serum	Osteoclastic enzymes
Formation				
OC	Osteocalcin	Automated Manual	Serum/plasma	Bone matrix proteins
ALP	Alkaline phosphatase	Automated Manual	Serum/plasma	Osteoblast enzymes
BALP	Bone-specific alkaline phosphatase	Automated Manual	Serum/plasma	Osteoblast enzymes
PICP	Propeptides of type I collagen: C-terminal	Manual	Serum/plasma	Collagen synthesis by-products
PINP	Propeptides of type I collagen: N-terminal	Automated Manual	Serum/plasma	Collagen synthesis by-products

Adapted with permission from Civitelli et al. 2009 [25]

2.2.5 Bone turnover: age-related transition

Ageing is associated with significant bone loss in both men and women [30]. Bone formation during childhood and adolescence exceeds that of bone resorption allowing for bones to grow. The rate of bone formation in children and adolescence exceeds that of bone resorption allowing bone growth through remodelling. In adulthood, bone growth ceases and the cycle of bone repair and formation is known as bone remodelling [6]. However, bone growth ceases in the adult skeleton leaving the process of bone repair and bone formation in adulthood to the 'bone remodelling' activity. Menopause in women has been associated with rapid trabecular bone loss especially in the vertebrae, pelvis and ultra-distal forearm [31] due to oestrogen loss. The process of bone resorption (2 weeks) occurs a lot quicker than bone formation (4 months) with the kinetic difference exacerbating bone loss during menopause [9].

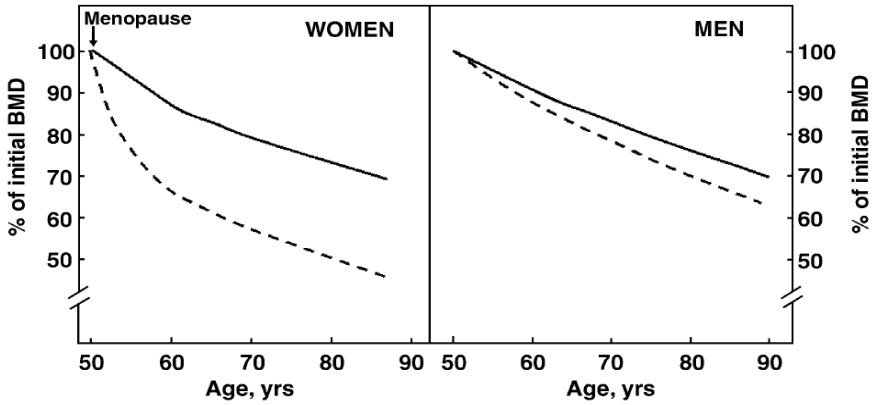

Figure 2-4 Patterns of age-related bone loss

Figure 2-4 depicts the patterns of bone loss in women and men based on multiple cross-sectional and longitudinal studies using the DXA. Dashed lines represent trabecular bone while solid lines represent cortical bone. Reproduced with permission from Khosla and Riggs, 2005 [31].

2.2.6 Postmenopausal osteoporosis

The onset of menopause causes oestrogen levels to decline rapidly which leads to increase RANKL production and this has been associated with increased bone resorption in postmenopausal women [32]. Osteoclasts are regulated by signalling molecules such as RANKL. The RANKL promotes the differentiation, activation and survival of osteoclasts via interactions with its receptor RANK [33]. On activation, osteoclasts resorb bone. Meanwhile, the hormone oestrogen has been reported to repress RANKL signalling and the downregulation of RANKL expression consequently leads to decreased activation of RANK and subsequent decrease in bone resorption. Oestrogen also stimulates the expression of osteoprotegerin (OPG) which inhibits the activity of RANKL by preventing interactions with its receptors [32, 33].

2.2.7 Biochemical bone regulation

2.2.7.1 Role of parathyroid hormone

The parathyroid glands produce parathyroid hormone (PTH) known as the main regulator of calcium and phosphorus levels in the blood. PTH also plays a significant role in bone remodelling. Low levels of plasma calcium stimulate the release of PTH by parathyroid glands and in the bone PTH stimulates the release of calcium and phosphate into the plasma.

In the bone, PTH binds to PTH receptor on the osteoblast which leads to the proliferation of the osteoblast and the inhibition of OPG production and effects. As a result, inhibiting the activity of RANKL [34]. In glucocorticoid-induced osteoporosis, PTH has been indicated as a significant contributory factor [35]. This most especially due to the relationship between hyperparathyroidism and PTH-induced bone loss which is as a result of vitamin D deficiency that is very common in the elderly.

2.2.7.2 Role of vitamin D

Vitamin D can either be synthesised in the human skin or from foods that contain Vitamin D_3. Vitamin D_3 synthesised in the skin after the conversion of 7-dehydrocholesterol (7-DHC) to pre-vitamin D_3 through actions of ultraviolet B rays at the wavelength 280 – 315nm [35]. Some foods such as milk, margarine, oily fish, bread and yoghurt are rich in vitamin D_3. Vitamin D is carried in the bloodstream to the liver, where it is activated and converted into the prohormone calcidiol (25-(OH)D). Circulating calcidiol may then be converted into calcitriol (1,25-$(OH)_2$ D_3), the biologically active form of vitamin D, in the kidneys (by 1-α hydroxylase). This form of vitamin D (i.e. calcitriol) promotes gut absorption of calcium in bone metabolism and therefore exerts regulatory effect on calcium balance. The primary action of 1,25-$(OH)_2$ D_3 is to promote gut absorption of calcium by stimulating formation of calcium-binding protein, calbindin, within the intestinal epithelial cells. It also promotes intestinal absorption of phosphate ions in the body [6]. Women with vitamin D levels of lower than 19 ng/mL had an increased risk for fractures vs women with levels of 28.2 ng/mL or higher. In adults with serum 25(OH)D levels of less than 10 ng/mL, vitamin D supplementation can improve skeletal muscle strength [35, 36].

2.2.7.3 Role of oestrogen

The decrease in the hormone oestrogen synthesis in women due to amenorrhoea (cessation of menstrual cycles) after puberty or before the onset of menopause has immense threat to bone health status. This as a result of the knowledge and insight into the actions of oestrogen deficiency on bone remodelling and therefore the pathogenesis of postmenopausal osteoporosis. Oestrogen is responsible for modulating the secretion of immune cell cytokines and growth factors found in the bone marrow and bone cells [37].

2.2.8 Factors affecting bone density

There are various factors that affect bone health and the risk of osteoporosis. For the purpose of this thesis, these factors will be classified into modifiable and non-modifiable factors. Aside from the impact of the non-modifiable factors, the role of an individual's lifestyle in modulating BMD is profound and cannot be underestimated.

2.2.8.1 Non-modifiable Factors

2.2.8.1.1 Age

Age is an unmodifiable factor that contributes to bone loss across all ethnicity. This is why nutrition education and awareness campaigns are important means of informing groups at risk in order to alleviate the burden of the disease. BMD is known to achieve peak by the age 30 to 35 years of age after which there is a decline of BMD with age in both men and women [38]. The importance and relevance of the peak bone mass (PBM) have been postulated that an increase of PBM by one standard deviation reduces risk of fracture by 50% [39]. Factors contributing to age-related bone loss include secondary hyperparathyroidism (due to prevalent vitamin D deficiency), gonadal sex steroid deficiency and the accumulation of bone marrow fat amongst others [40].

2.2.8.1.2 Sex

Another non-modifiable factor is sex. Studies have shown that women are at more risk than men with the risk for women increasing two-folds with the onset of menopause [30]. A study by Khosla et al. indicated that in comparison to women, men have a thicker trabeculae and less structural damage with ageing, concluding that trabecular volume is different between sexes [30]. This is narrowed down to the fact that men do not have the equivalent of menopause or oestrogen loss.

2.2.8.1.3 Race and genetics

Racial or ethnic differences and genetic disposition play a significant role in determining frame size and consequently bone strength. The Caucasian (White) race is well known as a risk factor for lower BMD while being an African (Black) is a protective factor for bone strength amongst both adults and children alike [41]. Large degrees of variation in individuals also exist within ethnic groups especially in relation to fracture risks.

2.2.8.1.4 Other factors

Individuals with fracture(s), a family history of osteoporosis, and those on medication due to some illnesses may be at a higher risk of becoming osteoporotic. Examples of medicines that could cause bone loss are glucocorticoids such as cortisone and prednisone used for treating arthritis, asthma and multiple sclerosis. Proton pump inhibitors, selective serotonin receptor inhibitors thiazolidinediones, anticonvulsants, aromatase inhibitors, androgen deprivation therapy, heparin, medroxyprogesterone acetate calcineurin inhibitors, and some form of chemotherapies have been indicated as deleterious to bone health [42].

2.2.8.2 Modifiable Factors

2.2.8.2.1 Physical activity

Physical activity and muscle strength have been related to bone strength. Lean mass is known to be an important factor contributing to a higher BMD [43]. Muscle strength generates weight-associated gravitational forces on the bone by imposing mechanical stress during discretionary physical activity. Burr et al. [44] expressed the relationship between grip strength and age in men and women, depicting that grip strength declines from the age of 40 years. Another study indicated that loss of muscle strength begins at the age 30 years [45]. Whichever way we look at it, reduced muscle strength contributes to bone loss during ageing and especially during post-menopause in women. During the growing age, accumulation of muscle mass is essential to protect against bone loss later in life. In addition, a research suggest that almost 1% of bone loss can be prevented or reversed with exercise training programs [46].

2.2.8.2.2 Weight

Individuals with lower body mass index (BMI) are more prone to lower BMD and therefore, osteoporosis and fragility fractures. A large BMI has been related to higher BMD due to the mechanical loading and skeletal muscle mass; likewise obesity has been linked to hyperinsulinemia, low-grade inflammation and consequently higher secretion of inflammatory cytokines at the cellular level leading to loss of bone [47]. Although BMI is an indicator of bone characteristics, a high body fat percentage may be considered the focus for osteoporosis risk [48]. This is due to the proposed mechanisms of action and interrelationship between osteoblasts and adipocytes originating from mesenchymal stem cells [49]. The adipose tissue is known to secrete/express molecules such as resistin, leptin, adiponectin and interleukin-6 [49]. Correspondingly, research suggests that high visceral fat and inflammation exerts negative effects on bone structure [50].

2.2.8.2.3 Alcohol abuse and smoking

A lifestyle of high alcohol consumption and smoking has been related to poor bone in both men and women, young and old alike. High consumption of 2-4 standard drinks per day has been linked to bone tissue damage while one drink for women and two for men are referred to as non-deleterious [51]. Similarly, smoking has been reported to be negatively correlated with bone health, most especially dose-dependently [52].

2.2.8.2.4 Diet

The contribution of diet to bone growth is profound and cannot be underestimated. Many studies have shown that food and nutrients are very important for bone density building. Meanwhile, more recently researchers have been looking at the nutrient patterns and dietary patterns as it occurs in foods in relation to bone health as is shown in the studies below.

2.2.9 Importance of selected nutrients on bone health

2.2.9.1 Calcium

Calcium is a major constituent of bone. It is the most abundant mineral in the body with about 920 to 1500 g in adults depending on age, race, gender and size of the human body [53]. It is well known that 99% of this calcium can be found in the teeth and bones with only 1% in the other cell types and tissues in the body [54]. Calcium is found in the bone as hydroxyapatite crystals [$Ca_{10}(PO_4)_6(OH)_2$] in the form of calcium phosphate which are deposited along collagen fibrils, along with a small component of calcium carbonate to provide structure and rigidity [53]. Dietary calcium is a nutrient that function based on threshold capacity. At suboptimal levels, the bone tissue's uptake of calcium is limited [55]. Thus, increasing calcium intake above the threshold does not result in more bone formation [53].

Calcium is also important for skeletal muscle and vascular contractions, vasodilation, intracellular signalling, nerve transmission and secretion of hormones [53]. Postmenopausal women are at risk due to hormonal changes that may affect bone mineralisation process. The elderly are at risk of bone degradation for various reasons which include medication interactions with dietary calcium absorption and low calcium intake over time [56]. Several long-term studies have been conducted on the relationship between dietary calcium intake, osteoporosis and fracture risk [55-57].

The requirement and bioavailability of calcium vary between individuals based on their genetic makeup, environment and life stage [58]. The adequate calcium intake for adults between 19 to 50 years of age is 1,000 mg per day. Foods such as milk, cheese and other dairy foods as well as soya beans, tofu and green leafy vegetables are rich sources of calcium.

2.2.9.2 Phosphorus

Phosphorus is a vital element necessary for multiple biological processes. Phosphorus is a necessary component of most enzymes and cellular messengers such as G-proteins and carbohydrate fuels. Phosphorus occurs mainly as phosphate esters and to a lesser degree as phosphoproteins and free phosphate ions in soft tissues and cell membranes. It is the second most abundant inorganic element in the body. About one-tenth of the phosphorus content in the extracellular fluid is bound to proteins while approximately one-third is known to be complexed with sodium, calcium, and magnesium, and the remaining is available as inorganic phosphate [59].

Phosphate (a predominantly intracellular anion) is the most abundant anion in the human body. Phosphate is mainly complexed with calcium in the form of hydroxyapatite crystal in the skeleton. The remaining phosphate will normally be present as amorphous calcium phosphate. Phosphorus is a critical component of virtually all enzymes, cellular messengers such as the G-proteins, and carbohydrate fuels.

Compounds containing phosphorus play an important role in the composition and the development of normal skeletal system and in the responsiveness to morphogenetic proteins e.g. bone morphogenetic protein 4. They are also essential for cell structure maintenance (in combination with calcium), generation of ATP in cellular metabolism (glycolysis, gluconeogenesis), energy metabolism, regulation of subcellular processes and in the maintenance of acid–base balance and bone/skeletal mineralisation. The deficiency of phosphorus can therefore lead to defective mineralization of bones. An important determinant of the renal synthesis of 1,25-(OH)2D and therefore its plasma concentration is phosphorus.

Nutritional requirements for phosphorus, like calcium are determined by bone criteria. The current New Zealand recommended dietary intake in adults and the elderly is 1,000 mg per day while that of the adolescents is 1,250 mg per day. However, unlike calcium, common foodstuffs such as meat, fish, eggs, cereals and vegetables are naturally rich in phosphorus. Phosphate salts, in particular, polyphosphates are used as additives in foods such as surimi, ham and processed cheese. Deficiency of phosphorus is therefore very rare in humans and supplementation is of no use and can be damaging unless if deemed necessary [60].

2.2.9.3 Protein

Protein makes up approximately half of the volume of the bone and roughly one-third of its mass however this bone protein matrix continuously undergoes both remodelling and turnover [60]. Daily dietary protein supply is required for the bone maintenance of the crosslinking of collagen molecules in the bones which involves posttranslational modifications of the amino acids. The anabolic drive of amino acids on the organisms is influenced and mediated in part by the stimulation of the growth factors such as insulin-like growth factor I (IGF-I). Research evidence has shown that the IGF-I increases bone mass by increasing the osteoblast activity and thereby the mineralisation of bone matrix and calcium absorption [60].

High intake of protein has been linked traditionally as a risk factor for osteoporosis. This can be described by the proposed mechanism that suggests increasing protein intake increases acid production and renal acid excretion due to protons release during oxidation of sulphur – containing amino acids such as cysteine, methionine and cystine [61]. However, more recent studies on the effects of high and low meat diet on body's calcium retention in healthy postmenopausal women found no difference in the retention of calcium. The recommended dietary allowance for protein is established as 0.8 grams of proteins per kilograms of body weight. This level is set for normal individuals and is sufficient to prevent diseases and deficiencies. Observations in young women have shown that various protein intakes from 0.7 to 2.1 kg/body weight indicates that the high level of protein intake increases bone resorption and urinary calcium excretion with no concomitant increase in bone formation, while the low protein intake decreases calcium absorption and increases PTH [62]. Meanwhile, the medium protein intake did not change any of the above factors. The medium level (1.1 g/kg/d protein) is higher than any of the current recommendations (0.8 g/kg/d protein).

2.3 The Role of Dietary Patterns in the Pathogenesis of Osteoporosis

Osteoporosis is a major public health concern in the ageing populations [63]. Nutrition is essential in promoting health and in the prevention of diseases. Many nutrients are known to interact with each other thereby influencing their bioavailability and absorption [64]. Several key nutrients are known to affect the bone mineral content and BMD. These nutrients, however, are present in foods and dietary patterns, therefore there is a need to study the diet in its entirety. Dietary patterns have been known to be associated with some chronic diseases such as diabetes and cardiovascular disease [65]. On the other hand, the overall effects of dietary choices on bone health is not well understood and needs further research.

There are two main holistic approaches or methodologies to describing and quantifying dietary patterns/habits: 1. The *a posteriori* (data driven) dietary pattern approach i.e. the use of statistical methods such as principal component analysis (PCA) or factor analysis, reduced rank regression (RRR), cluster analysis and partial least squares to generate dietary patterns from data collected; and 2. The *a priori* dietary pattern approach i.e. the use of created or predefined dietary indexes on the basis of existing knowledge in nutrition usually complying with dietary guidelines and recommendations [66].

2.3.1 *A Priori* dietary patterns

There are a number of different types of dietary indexes used for scoring dietary intake patterns in study populations which include the Mediterranean diet score, Healthy Eating Index (HEI), Alternative Healthy Eating Index (AHEI), Diet Quality Index-International, Food Group Intake Pattern, Dietary Diversity Score, Oslo Health Study Index (OHS), Dietary Approaches to Stop Hypertension (DASH), Recommended Food Score (RFS), Korean Diet Score, Mean Nutrient Adequacy Ratio, Health Diet Indicator, Dietary Inflammatory Index, Nutritional Risk Score and Bone Mineral Density (BMD) Diet Score. These indexes are important for scoring *a priori* studies.

2.3.2 A *Priori* dietary patterns in Children and Adolescents

A priori dietary pattern was used to see if there is a relationship or differences in the BMD of adolescents between 13 and 17 years of age and no difference was observed [67]. However, the male adolescents aged 13 years adhering to a modified Mediterranean diet score for children and adolescents was associated with higher distal radius BMD. Another clinical trial observed an increase in the urinary bone resorption biomarker and improved calcium absorption and retention in comparison with the baseline measurements over 28 days with a Mediterranean based diet [67].

Adherence to the OHS dietary index and DASH dietary pattern were not associated with their BMD [68]. One possible explanation could be due to the main limitation of the *a priori* dietary patterns approach which is related to the use of dietary guidelines that generally are not disease-specific whereby adherence to them reduces the risk of some diseases but not others [64]. It could also be as a result of cofounders not accounted for such as body size and physical activity.

2.3.3 *A Priori* studies on bone mineral status In Adults and the Elderly

Oslo Health Study Index was used in a cross-sectional study in Norway to investigate the positive effect of fruit and vegetable and negative effect of soft drinks on bone density in a population-based study. The study recruited 1,255 women and 871 men aged 30-60 years with result showing an inverse association between soft drinks intake and distal forearm BMD [69].

Findings by the vitamin D status (cross-sectional) study in University of Massachusetts, United States, suggest that existing diet quality scores were not appropriate for peak bone mass studies indicating that this could be as a result of insufficient weight allocated to foods and nutrients that are of importance to bone health. Result from the RFS a simple measure of diet quality based on consumption of recommended food items, and the AHEI, a relatively complex index of diet quality incorporating a variety of dietary components, were not associated with BMD parameters [70].

On the other hand, results of adherence to alternate Mediterranean (aMed) score (an adapted principle of the traditional Mediterranean diet for non-Mediterranean countries) in a community-based Chinese adult aged 40-75 years indicated favourable associations with BMD at all sites. In this cross-sectional study, better adherence to the Mediterranean diet (i.e. higher aMed scores) was found to be favourably associated with BMD in middle-aged and elderly Chinese populations [71].

The FLAMENCO project was a cross-sectional study comprising of 197 perimenopausal women aged 45-60 years [72]. In Spain, the main findings of adherence to Mediterranean diet when considered in association with physical fitness, body composition and cardiometabolic markers indicated that neither cardiorespiratory fitness, flexibility, motor agility, cardiometabolic markers (blood pressure, lipids, fasting glucose or C-reactive protein), nor the Mediterranean diet were associated with BMD in perimenopausal women. By contrast, skeletal muscle strength, body weight, BMI and lean mass were associated with BMD in this population'. Lean mass was found as the only factor independently associated with BMD. In two age groups of Spanish women (100 premenopausal 34±7y, pre-M and 100 postmenopausal 54±6y, pos-M) aged 18-65 years, Mediterranean diet measured as Mediterranean Diet Score was associated with higher BMD in all subjects. BMD was measured through DEXA of the calcaneus [73].

However, in Northern Ireland, a unique cross-sectional study examined the relationship between bone mineral status, a posteriori PCA-derived dietary patterns and a priori dietary scores in younger adults aged 20-25 years. They found no association neither with the MDS (rich in fish and olive oil and low in meat and meat products), nor the Nutritional Risk Score and BMS. Although, for women, the 'Nuts and Meat' dietary pattern was associated with greater FN BMD and femoral neck bone mineral content (FN BMC) in unadjusted and adjusted analyses; also, women with the highest Dietary Diversity Score had greater FN BMD. The authors have also expressed concern that currently there are no bone-specific a priori scores available [74].

Similarly, no association was found in a Greek women's cross-sectional study of 196 pre- and perimenopausal women aged 48 ± 2 y. However, adherence to a dietary pattern with the features of the Mediterranean diet, i.e., rich in fish and olive oil and low in red meat and products, was found to be positively associated with lumbar spine BMD and total body bone mineral content (TBBMC). They described the Mediterranean diet as a pattern characterised by high intake of vegetables, fruits, legumes and non-refined cereals, fish, and olive oil, low intake of red meat and full-fat dairy products, and moderate consumption of poultry and alcoholic beverages [75].

More *a priori* dietary patterns were conducted for BMS including BMD Diet Score and Healthy Diet Indicator (HDI) by Jonge et al. in The Rotterdam Study in Netherlands [76]. The BMD-Diet score and the DASH-Diet score share common components, namely fruits, vegetables, fish, and whole grains which are favourable (high-BMD) food groups and (red) meat which is an unfavourable (low-BMD) food group [76]. The DASH-Diet score, similar to the BMD-Diet score also uses a more specific definition of low-fat dairy products as favourable components. The BMD scores were based on ascending values of quartiles of high BMD components (fruits, vegetables, dairy products, whole grain products, fish and legumes and beans) and descending values of quartiles of low BMD components (red meat, processed and organ meat and confectionary).

The results of the study found BMD-Diet score was associated with high FN BMD while HDI was directly associated with FN BMD, but 3 times weaker than BMD Diet Score [76]. Conversely, the association between Dietary Inflammatory Index (DII) and BMD in a sample of 160 postmenopausal Iranian women aged 50-85 y indicated no association in the FN. However, the intake of this pro-inflammatory diet was associated with a risk for lower BMD in the lumbar spine [77].

2.3.4 *A Priori* studies using biomarkers In Adults and the Elderly

The employment of the Healthy Eating Index 2005 (a diet quality assessment tool) to assess the association between diet quality and bone turnover markers (BTMs) by Hamidi et al. showed no relationship between the BTMs and HEI-2005. The cross-sectional NHANES 1999-2002 study was carried out involving 827 postmenopausal women aged ≥45 years [78].

In another cross-sectional study, the Korean Diet Score (KDS) was developed using the Nutritional recommendations in the Food Guidance System which are based on the Dietary Reference Intakes for Koreans (KDRIs) and Dietary Guidelines for Koreans. In the study (4th Korean National Health and Nutritional Examination Survey 2007 and 2008) of 5320 women and men aged 30-80 years, KDS was inversely associated with the risk of osteoporosis [79].

Furthermore, work done by Go et al. utilising the fifth Korean National Health and Nutritional Examination Survey (2010) found no association with the nutrient adequacy ratio of the dietary quality analysis. Using the Dietary Diversity scores the results were inversely associated with the risk of osteoporosis and osteopenia which they based on WHO criteria. Under the Dietary Pattern Analysis, there was no association with the Food Group Intake Pattern however milk, anchovy, and sea mustard were inversely associated with the risk of osteoporosis and osteopenia in the Assessment of Calcium source consumption [80]. The cross-sectional study consisted of 847 postmenopausal women whose food group intake patterns were analysed using food consumption data and foods eaten classified as Grain, Meat, Dairy, Vegetable, and Fruit [80].

2.3.5 *A Priori* studies on fractures In Adults and the Elderly

In a Three-City (3C) population-based longitudinal study, adherence to Mediterranean diet was not associated with decreased risk of fractures amongst French older population aged ≥67 years at baseline (consisting of 932 women and 550 men). However, the results indicated that lower intake of dairy products was associated with increased risk of fracture at any of the sites especially with a double risk of wrist fracture but not at other sites in fully adjusted models for body composition. Doubled 8-year risk of hip fracture was associated with greater fruit consumption. A ratio of MUFA/SFA higher than 0.8 was associated with a lower risk of vertebral fracture once the model has been adjusted for certain variables and all other dietary components in the (Mediterranean diet) MeDi score [81]. The study however failed to find a conclusive association between these variables.

On the other hand, in a much larger population (139,981 women and 48,814 men aged 35-70 years at baseline), the European Prospective Investigation into Cancer and Nutrition study conducted a longitudinal study using a modified version of the Mediterranean diet score. The result indicated that higher vegetable and fruit intake was associated with lower hip fracture incidence. However, the higher meat consumption was associated with higher hip fracture incidence [82]. Meanwhile, the higher the ratio of the sum of mono- and polyunsaturated lipids (unsaturated lipids) to saturated lipids, the lower the incidence of hip fractures, and further analysis showed that the increased consumption of saturated lipids was associated with higher hip fracture incidence. Overall, the inverse association between adherence to modified Mediterranean diet and hip fracture incidence was more pronounced and evident in men than in women [82].

In a recent longitudinal cohort study including Swedish 37,903 men and 33,403 women Byberg et al. aimed to establish the association between the rate of hip fracture incidence and adherence to Mediterranean diet. The outcome of this research indicated an inverse association between modified Mediterranean diet and hip fracture similarly in men and women alike [83].

In China, Zeng et al. assessed and compared the efficacy and validity of four widely used diet quality scoring systems in 549 pairs of women and 177 pairs of men age-matched and gender-matched; aged 55-80 years, in a case-control study. They concluded that lower diet-quality scores of HEI-2005, aHEI, DQI-I, and aMed were associated with a similar and greater risk of hip fractures and suggested that aMed is the best scoring system in terms of performance and user-friendliness [84]. Results from a prospective population-based cohort of 63,257 Chinese men and women (pre and postmenopausal) aged 45-74 years in the Singapore Chinese Health Study show that in both genders, higher scores for the vegetable-fruit-soy pattern and the AHEI 2010 were associated with lower risk of hip fracture dose-dependently [85]. They concluded that since plant-based foods such as soy, fruits and vegetables substantially lower the risk of hip fracture and they might prevent osteoporotic hip fractures [85].

During a recent longitudinal (the Women's Health Initiative) study involving a 19-21 years follow-up, the results indicated an inverse association between Mediterranean diet and the risk of hip fracture. This observational study in the US based its main outcome and measure on total and hip fracture of a total of 90,014 postmenopausal women, aged 50-79 years at baseline [86]. As can be noted from all of the research work adherence to a healthy dietary pattern supports and play a role in the maintenance of bone health in postmenopausal women.

2.4 *A posteriori* dietary patterns

These types of data-driven dietary patterns which are normally generated from multivariate statistical methods highlight the peculiarities of the individuals' dietary choices. They are therefore not limited to the use of a predefined dietary indexes; however, these studies undertake exploratory factor analysis that involves subjective decisions.

2.4.1 *A posteriori* dietary pattern studies that used factor analysis for BMD/BMC

In a cross-sectional study of 291 premenopausal farmwomen aged 40-55 y, 4 dietary patterns were identified. Distal radius and ulna BMD and BMC were measured by DXA. The Japanese Multi-centered Environmental Toxicant Study (JMETS) found the healthy (featuring high intakes of green and dark yellow vegetables, mushrooms, fish and shellfish, fruit, and processed fish) pattern was directly associated with BMD. On the other hand, Western dietary pattern was inversely associated with BMD, however, not significant [87]. Probably, due to the sample size.

Similar result was found in another cross-sectional research work conducted in Greece on 196 pre-, peri- or postmenopausal women aged 48±2 y that measured the LS BMD and total body BMC by DXA. Kontogianni et al. identified 10 dietary patterns and concluded that the patterns characterised by high consumption of fish and olive oil and low intake of red meat was positively and directly associated with LS BMD (P = 0.017) and total body BMC (P = 0.048), after they have controlled for confounders [75].

The Canadian Multicenter Osteoporosis study determined the relationship between dietary patterns and FN BMD in 4,611 women and 1,928 men participants aged ≥25 y at baseline in a population-based longitudinal cohort. The study identified two dietary patterns namely (with analogous) nutrient dense (prudent) high in fruits, vegetables and whole grains and energy-dense (Western) comprised of soft drinks, potato chips and French fries, including meats (hamburger, lunch meat, bacon, hot dog, and sausage), and desserts (chocolate, doughnuts, ice cream) [88]. The results indicated that Western dietary pattern was significantly associated with a decrease in FN BMD for men 50+ years and postmenopausal women whereas an increase in the prudent dietary pattern was associated with an increase in FN BMD in men aged 25-49 years old [88]. This study however contains some missing information (FFQ and BMD) as well as the inability to measure absolute energy intake due to lack of specified portion size in the FFQ.

The cross-sectional analysis of a co-twin controlled study with linear regression modelling in United Kingdom with >2,000 postmenopausal women aged 56.3 ± 11.9 y yielded 5 patterns of dietary exposure. They observed that high traditional 20th-century English dietary pattern, characterised by high intakes of fried fish, fried potatoes, legumes (e.g., baked beans), red and processed meats, savoury pies, and cruciferous vegetables (egg, cabbage and cauliflower) was inversely associated with FN BMD (hip neck). Meanwhile, alcohol intake from wine (and not beer and spirits) was directly associated with spine BMD. The findings indicate that consumption of 1 unit (standard drink) alcohol/d increased bone density in the spine by 0.03 g/cm^2 [89].

In a research contribution by McNaughton et al., they evaluated the LS BMD, total hip BMD and total body BMC by DXA and assessed the dietary patterns of 527 Australian women aged 18–68y who participated in the Twin and Sister Bone Research Program by the use of a 4-d food diary. The result of their analysis revealed 5 dietary patterns of which pattern 1 (high intake of refined cereals, soft drinks, fried potatoes, sausages and processed meat, vegetable oils, beer, and takeaway foods and low consumption of other vegetables, vegetable dishes, tea, coffee, fruit, wholegrain breads, and breakfast cereals) was inversely associated with total body BMC.

Pattern 4 of the Twin and Sister Bone Research (high consumption of legumes, seafood, seeds, nuts, wine, rice and rice dishes, other vegetables, and vegetable dishes and low consumption of bacon and ham) was directly associated with BMD. They also found an inverse association between dietary pattern 5 and LS BMD [90].

In a group of 3,236 postmenopausal Scottish women aged 50–59 y from the Aberdeen Prospective Osteoporosis Screening Study, Hardcastle et al. identified five dietary patterns of which three were found to be correlated with bone health. The healthy dietary pattern with high positive loadings for fruit, vegetables and rice or pasta was associated with decreased bone resorption. Moreover the processed foods dietary pattern, with cakes and desserts having the strongest positive loadings of 0.39 and 0.37, respectively and the snack foods dietary pattern included positive loadings for confectionery, crisps or nuts and sauces were both inversely associated with FN BMD and LS BMD [91]. The study however relied on reported FFQs, had a small strength of association between the dietary patterns and bone biomarkers and was another cross-sectional study therefore causality cannot be proven.

In an investigation into the association and interactions of antioxidant vitamins and carotenoids with bone health in 293 postmenopausal Japanese women aged 60.2±6.2 y presented an interesting result. Three dietary patterns of antioxidant vitamins and carotenoid intakes were identified 1) Carotene 2) Retinol and 3) β-cryptoxanthin of which the Retinol dietary pattern consisting of high intakes of preformed retinol, zeaxanthin, and vitamin E, was positively associated with the risk for low radial BMD. Meanwhile β-cryptoxanthin dietary pattern characterised of high intakes of β-cryptoxanthin and vitamin C was negatively associated with low radial BMD [92].

However, there were a few limitations in the study; the survey used did not include portion size questions because absolute nutrient intake cannot be estimated from FFQ also the method of measurement requires femoral neck or lumbar spine BMD but the study evaluated the radial BMD at 1/3 (33%) of the forearm length measured from the styloid process on the ulna [92]. In addition, it was a small sample-sized cross-sectional study, therefore causality cannot be assumed.

Amongst young adults aged 20-25 y in Ireland, Whittle et al. investigated the relationship between dietary patterns and bone health using *a posteriori* and *a priori* methods [74]. In the Northern Ireland Young Hearts Project, they measured the FN BMD, BMC and LS BMD and BMC by DXA and they also measured the dietary assessment with the 7-day diet history. In a sample of 238 women and 251 men they came up with four dietary patterns; 1) Healthy, 2) Traditional, 3) Refined in men and Nuts and meat in women, 4) Social. Pattern 3 in men was inversely associated with FN BMC while pattern 3 in women was significantly associated with FN BMD and FN BMC [74]. However, this is yet another cross-sectional study with a modest amount of sample.

A study conducted in Tehran, Iran on 160 menopausal women aged 50–85 y revealed six dietary patterns. Pattern 1 characterised by high intakes of high-fat dairy products, organ meats, red or processed meats and non-refined cereals. Pattern 2 consisted of French fries, mayonnaise, sweets and desserts, and vegetable oils. Pattern 3 was high hydrogenated fats, pickles, eggs and soft drinks, while pattern 4 was mainly high intakes of vegetables, low-fat dairy products, fruits and fruit juices, legumes and fish, and low intakes of salt. Pattern 5 was characterised by high condiment and potatoes and low refined cereals. Pattern 6 consists of high consumption of snacks, tea and coffee, poultry and nuts [93].The result of this study by Karamati et al. showed that there was an inverse relationship/association between pattern 1 and LS BMD as well as between pattern 2 and FN BMD [93]. The cross-sectional study however cannot be generalised and/or representative of the population due to the sample collection method.

Another study by the same authors generated 3 nutrient patterns; pattern 1 is high in folate, total fiber, vitamin B-6, potassium, vitamins A, C and K, β-carotene, magnesium, copper and manganese; pattern 2 is abundant in vitamin B2, protein, calcium, phosphorus, zinc, vitamin B12, and vitamin D and low in vitamin E; pattern 3 is characterized by high total fat, MUFAs, SFAs, PUFAs, and low carbohydrates and vitamin B-1. Their findings indicated a positive association between pattern 1 and lumbar spine BMD but not at the femoral neck [94]. These results are similar to that of New et al. which show that LS BMD was higher with high intake of potassium, magnesium, vitamin C, fiber, and zinc in premenopausal women by using dietary intake [95]. It can also be seen to be in accordance with the results of Sugiura et al. in terms of the relationship between the antioxidant nutrients (abundant in fruits and vegetables) and risk of low radial BMD among Japanese postmenopausal women [92].

A 2-year prospective study investigated the relative contribution of lean mass, fat mass and dietary patterns on BMD in post-menopausal women aged 50-65y at baseline [96]. The research work on 282, 212 and 202 women at baseline, year 1 and year 2 respectively generated six dietary patterns of which the intake of the cereal grains-fruits pattern was associated with decreased hip and LS BMD and high intake of milk-root vegetables was positively associated with BMD of hip [96]. The authors also concluded that lean mass and not necessarily fat mass, years since menopause or age of menophania is the best determinant and predictor of BMD, nonetheless, the study was conducted on Chinese only participants were from one clinic [96].

In Korea, the Healthy Twin Cohort study by Shin et al. conducted on 1,818 subjects (716 men, 1,102 women) aged ⩾30 years, used 3-day food record to evaluate the associations between dietary patterns and BMD. Four dietary patterns were identified (Rice and kimchi; Eggs, meat and flour; Fruit, milk and whole grains; and Fast food and soda).

In the Healthy Twin Cohort study, intake of fruit, milk and whole grains was correlated with a decreased risk of low total body BMD in both men and women and was directly correlated with whole arm, leg and total body BMD in women and whole leg, pelvis and LS BMD in men [97]. Their study also found intake of rice and kimchi to be directly correlated with whole arm BMD in both men and women [97]. These relationships however lack causality as this was another cross-sectional study design.

Furthermore, researchers in Brazil reported a study on 156 osteoporotic postmenopausal women aged ≥45 y investigating associations between dietary patterns and BMD [98]. Five dietary patterns were observed namely; 'healthy', 'red meat and refined cereals', 'low-fat dairy', 'sweet foods, coffee and tea' and 'Western'. The results indicated that 'Sweet foods, coffee and tea' dietary pattern high in sugar and sugary products was inversely correlated with femoral neck and total body BMD in this population [98].

2.4.2 *A posteriori* dietary pattern studies using factor analysis for bone biomarkers

Some of the studies above have included research work on bone biomarkers. For example, in the Aberdeen Prospective Osteoporosis Screening Study, aside from investigation of the BMD, the study also investigated the bone resorption and formation biomarkers. They found that the intake of the healthy food pattern was associated with decreased bone resorption biomarkers [91]. Likewise, the Canadian Multicentre Osteoporosis Study found the intake of the prudent dietary pattern was associated with decreased CTX in women and PTH in men while the intake of Western dietary pattern was associated with higher bone-specific alkaline phosphatase (BAP) and lower 25-OH-D in women and higher CTX in men [99].

2.4.3 A *posteriori* dietary pattern studies reporting the risk of osteoporosis

Few research studies have reported risks of osteopenia and osteoporosis using *a posteriori* dietary pattern. An example is the Korean Genome Epidemiology Study (a 4y longitudinal follow-up study), involving 1,464 postmenopausal women. Of the three dietary patterns (Traditional, dairy and Western) generated, intake of dairy dietary pattern was associated with low risk of osteoporosis meanwhile intake of traditional and Western dietary patterns was significantly associated with the risk of osteoporosis. Whereby osteoporosis incidence was by quantitative ultrasound T-score at the midradius and tibia shaft by ultrasound [100].

In a cross-sectional study, the Korean Health and Nutrition Examination Survey 2008-2010, 3,735 postmenopausal women aged 64±9y were investigated. Osteoporotic investigations were by LS and femur BMD T-score by DXA. The study resulted in 4 dietary patterns, out of which, the 'dairy and fruit' dietary pattern was associated with a decreased risk of osteoporosis of the lumbar spine. On the other hand, the 'white rice, kimchi and seaweed' dietary pattern was negatively associated with bone health [101].

A cross-sectional study on College freshmen in China investigated osteopenia and osteoporosis by speed of sound (SOS) T-score on the right calcaneous by ultrasound amongst 1,319 men aged 16-20y (18±1y). Their investigation yielded 4 dietary patterns of which the 'calcium' and the 'Chinese traditional' dietary patterns were inversely associated with the risk of osteopenia and osteoporosis [102].

To prevent metabolic diseases such as osteoporosis, it is important to better understand the role of diet on gut microbiota due to its central role on an individual's health. Dietary patterns studies are important, however, studies of the gut microbiome in relation to diet is paramount for bone health. This brings about the significance of the investigation of the gut microbiome and its analysis in the research into bone health status.

2.5 Gut microbiome as a target in the pathogenesis of osteoporosis

2.5.1 The Gut Microbiota

The density, diversity and activity of the gut bacteria can be modulated by both external factors (diet, prebiotics, probiotics, antibiotic usage, illnesses, lifestyle and living environment) and internal host properties (age, genetics, stress, physiological processes) [96]. The human gut microbiota confer benefits such as energy and nutrient extraction from the diet, production of vitamins, metabolic function, protection from pathogenic organisms and regulation of innate and adaptive immunity [103]. It has been linked to alterations in the microbiome such as Crohn's disease, inflammatory bowel diseases, obesity, malnutrition, metabolic disease, cancer and cardiovascular disease [103]. However, insight into the relationship between the gut microbiome and human health might help us explore the possibility of new probiotic and prebiotic supplementation and novel strategies in the treatment and management of a wide variety of human diseases [104] especially osteoporosis.

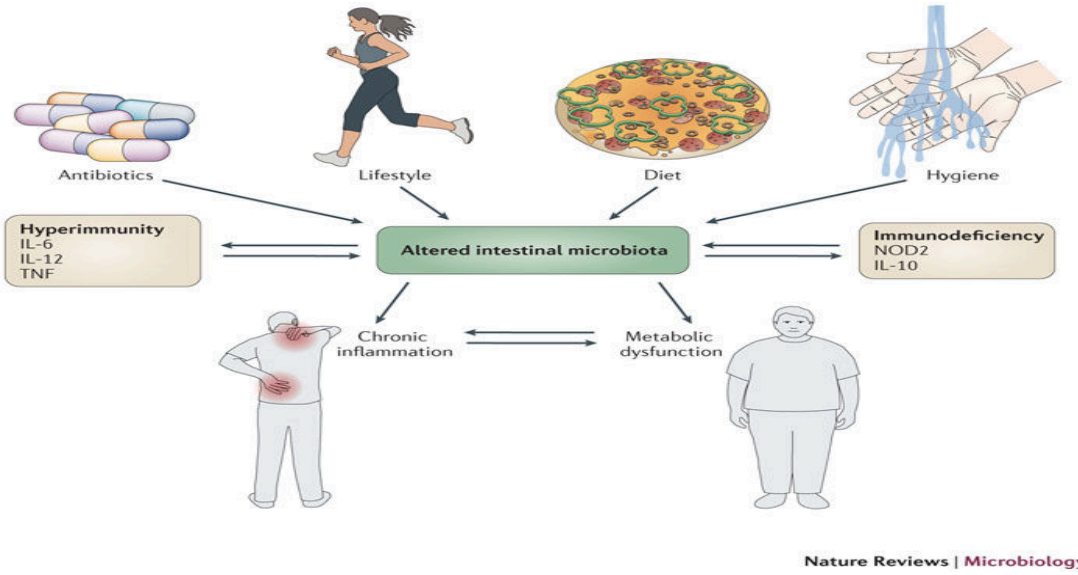

Figure 2-5 Intestinal microbial composition and the effects of dysbiosis on host health

Reproduced with permission from Nature Reviews Microbiology and Sommer and Bäckhed, 2013 [105].

The human gut is made up of epithelial cells, lamina propria and the *muscularis mucosae* which is home to about 30^{14} microbes of 5000 species and 5 million genes (known collectively as metagenome). For quite some time, it has been known that over 90% of the cells in the human body are microbes, located principally in the distal gut [106]. Normally, the human host is colonized by bacteria at the birth canal from the mother, as well as the environment. At first, the gut microbiome is varied but stabilises towards an adult-like configuration during the 3-year neonatal period after birth [107]. During this time, it has been noted that the immune system rapidly matures under the influence of the gut microbiota and other environmental factors such as diet, antibiotic treatments, gut infections and breast feeding. However, the composition of the gut microbiota changes with age and varies amongst older individuals age >65 years old [108, 109].

During homeostasis, the relationship between the host and the resident microbes is at an equilibrium; providing a situation that protects against invading pathogens and helps food digestion. Nevertheless, under compromised and/or pathological conditions that allows leakage of bacteria and entry from the gut lumen into host tissues and circulation and cause diseases [107]; a process known as dysbiosis. The composition of the gut microbiota can be altered by pathogens, antibiotic treatments or dietary changes which can shift its composition and thereby disturbing the balance in the metabolic and immune regulatory networks that normally supress/restrain inflammation [107].

The development of efficient and effective sequencing technologies and the use of many gnotobiotic (the germ free technology) animals have made it possible for the characterisation, composition and effects of the gut microbiota [107]. Methods have been largely limited to the use of faecal sample analysis in humans because of ethical issues around gut probing.

2.5.2 The Immune system regulated by the gut microbiota

The immune connection in bone metabolism indicates that the skeleton besides structural support, serves as a niche for mesenchymal stromal cell derived bone forming osteoblasts (OBs) and bone resorbing osteoclasts (OCLs) generated from hematopoietic stem cells. T cells originates from hematopoietic stem cells. The OCLs are derived from a lineage that determines whether their myeloid precursor cell will differentiate into either a macrophage, myeloid dendritic cell or an OCL. The appearance of a macrophage colony stimulating factor (M-CSF) results in increased proliferation and survival of nuclear factor -κB (RANK) in OCL precursor cells [107].

In a study involving the discordant or concordant for Crohn's disease in monozygotic twins, there was an increased ratio of an adherent invasive *Escherichia coli* to *Faecalibacterium prausnitzii* in the diseased twins. The anti-inflammatory function of *F. prausnitzii* showed that the ratio of pathogenic to more protective species is of significance, indicating that lower amounts of *F. prausnitzii* and higher amounts of *E. coli* are indicative of ileal Crohn's disease [110]. Faecal microbiota profiles in patients with Crohn's disease in a large multicentre analysis predicting disease activity was identified. During the active phase of Crohn's disease, the genus *Bifidobacterium* was decreased and it was increased to healthy levels during the remission phase [111]. *Bifidobacterium* genus represents species of the normal inhabitants of a healthy human gut and alterations in gut *bifidobacteria* levels, or species composition, are sometimes present in the cases of gut microbiota dysbiosis [112]. Furthermore, in a study that evaluated the immunomodulatory properties conducted on Crohn's disease patients at the time of surgical resection and 6 months later by fluorescence *in situ* hybridation analysis, *F. prausnitzii* was shown to exhibit anti-inflammatory effects, partly due to secretion of metabolites blocking the activation of NF-κB and the secretion of interleukin-8 (IL-8), thereby decreasing the proinflammatory colonic cytokine synthesis and inducing the secretion of the anti-inflammatory cytokines [113].

2.5.2.1 Probiotics

Probiotics have been defined as "live microorganisms that when administered in appropriate amounts can provide certain health benefits to the host" which by virtue of being microorganisms, have been studied for their health benefits and immuno-modulatory effects [114]. As a result, they have been generally found to be of benefit for the immune system and inflammatory conditions.

Research has signified that probiotic supplementation enlarged bone mass in the tibia of chickens treated for 6-weeks diet supplemented with or without probiotic strains. Since the gut microbiome is known to modulate bone loss in sex-steroid-deficient female mice, the hypothesis is that treating with probiotics may protect from ovariectomy-induced bone loss [115]. The drinking water form of probiotic strains of a single (*Lactobacillus paracasei* DSM13434; *L.paracasei*) or a mixture (*L.paracasei* DSM13434, *Lactobacillus plantarum* DSM 15312, and *L. plantarum* DSM 15313; L. mix) which was selected as a result of their anti-inflammatory properties given during a 6-week period initiated prior to ovariectomy were shown to protect the mice from bone loss and bone resorption [116, 117]. Further mechanistic studies showed that these probiotic treatments could also decrease the expression of two inflammatory cytokines TNFα and IL-1β, and proliferate the expression of osteoprotegerin (OPG) which is a potent inhibitor of osteoclastogenesis in the bone of ovariectomised mice [118].

Britton et al. 2014 reported the effect of the anti-TNFα activity of *Lactobacillus reuteri*, a commensal bacterium on ovariectomised mice. It was found that *L. reuteri* secreted beneficial immunomodulatory factors that altered the gut microbiome composition and prevented the ovariectomised-induced trabecular bone loss and TNF-mediated bone resorption [118]. Furthermore, the treatment restrained the ovariectomised-induced rise in bone marrow CD4+ T cells, suggesting that immune status in the bone can be modulated by the gut microbiome and as a result affect OCL-mediated bone resorption.

Oral intake of *L. reuteri* treatment resulted in a decrease in gut inflammation and an increase in trabecular bone mass in another study involving gonadal intact male mice [119]. The authors claim that the most probable mechanism by which the bone mass is affected by the gut microbiota involves altered systemic bone marrow, immune system, consequently, regulating osteoclastogenesis [119]. The authors of this study argue that the gut microbiota may serve as a novel therapeutic target for treatment or prevention of osteoporosis due to the fact that studies have shown that the gut microbiota is a regulator of host metabolism, immune status, and bone mass which in turn regulates osteoclastogenesis. Molecules produced (metabolites) by the gut microbiota can be both beneficial and/or harmful and are known to interfere with the endocrine cell permeability, enteric nervous system and the immune system in the gut. At homeostasis, the gut microbiota introduces a colonisation resistance to provide a balance for the epithelial and immune cells in order to protect the host from invading bacteria, viruses and other possible class of pathogens. Disturbances can be caused by antibiotic treatment, diets or pathogens resulting in inflammation, dysbiosis and tissue destruction [107].

At menopause, oestrogen deficiency that occurs results in prolonged increased formation and prolonged survival of OCLs causing increased production of cytokines promoting osteoclastogenesis and oestrogen's direct effect on OCLs. Research have shown that low-grade inflammation has an effect on bone turnover physiological status and ultimately pathological skeletal conditions such as osteoporosis. An estimate of low-grade systemic inflammation moderately elevated serum levels of high sensitivity C-reactive protein (hsCRP) have been recorded to be associated with low bone mass and increased fracture risk [107]. In conjunction with these data, a decrease in bone resorption markers in early postmenopausal women was noticed as a result of a blockade of the inflammatory cytokines TNFα and IL-1.

According McCabe et al., previous research has indicated that inflammation in the bone marrow contributes to bone resorption due to gut inflammation and ovariectomy. Increased levels of activated T cells have been hypothesised to lead to enhanced expression of TNFα in the bone marrow. TNFα stimulates the activity of osteoclastogenesis which disrupts the normal bone formation and resorption balance [114].

Furthermore, mice expended of T cells *in vivo* when treated with anti-CD4 and anti-CD8 antibodies were protected against ovariectomy-induced bone loss. This mechanism which involves an upregulation of TNF-producing T cells in the bone marrow of ovariectomised mice, further argues for a role of T cells and T cell-produced cytokines in bone turnover. [107].

Despite the large number of studies linking alterations in the gut microbiota to inflammatory diseases, it is still unclear whether these alterations are the cause or consequence of these diseases. Autoimmune conditions and gut-associated inflammatory disorders have therefore been associated with low bone mass, suggesting a connection between the gut and BMD [107]. An increasing number of studies have suggested that there are additional ways to regulate bone health.

2.6 Prebiotics and Mineral Absorption in bone health

Bone is a metabolic organ that the gut microbiome has been known to regulate by aiding in the absorption of the key bone minerals. Nutrition is known to be a modulatory factor for bone accrual as well as postmenopausal bone loss although genetics is the major determinant of peak bone mass. The gut microbiota plays a role in the absorption of minerals including calcium, magnesium and phosphorus [120]. Inulin, oligofructose and galactoologosaccharides are the most intensively studied prebiotics regarding the mineral absorption and retention. Prebiotics have been defined as nondigestible fermentable food ingredients that beneficially affect the host by selectively stimulating the growth and/or activities of one or more bacteria (probiotics) in the gut thereby exerting a health-promoting effect [121].

2.6.1 Prebiotics

Prebiotics comprise of a large group of non-digestible oligosaccharide that typically contains 2-10 sugar subunits but can usually have more than 60 subunits. These non-digestible oligosaccharides are of low molecular weight and generally include stuctures such as polydextrose, fructo-oligosaccharides, xylo-oligosaccharides (from xylan by hydrolysis), galactooligosaccharides (produced from lactose by transglycosylation), and soybean oligosaccharides (from soybean whey by extraction) [114]. The prebiotics with longer chain >10 subunits include inulin (contains fructo-oligosaccharide, from chicory root by extraction) and resistant starches.

Bacteria are responsible for expression of various genes modulated by changes in the environment. Many of these genes are responsible for encoding enzymes that are involved in producing metabolites such as short chain fatty acids (SCFA i.e. acetate, propionate, and butyrate) and some organic acids (e.g., lactate), branch-chain fatty acids, bile acid derivatives as well as vitamins. There are two mechanisms by which SCFA can affect calcium absorption which includes 1. by increasing the acidity in lumen of colon and caecum which activates the H/Ca ion exchange also enhancing calcium solubility; 2. by increasing the caecal villi structures (the ceacal weight) thereby maximising the surface area and calcium absorption [114].

Bacterial action, the generation of metabolic products is reliant on substrate availability which is partly provided by available substrate in the gut lumen. Prebiotics are therefore important elements that can be used to improve/modify the metabolites generated by the gut microbiota [114]. However, prebiotics can still have immunological and anti-pathogenic effect irrespective of metabolite production [122]. Prebiotics are components in asparagus, chicory, garlic, leek, Jerusalem artichoke, dandelion greens, banana, onion, and bran etc.

2.6.2 Mineral absorption: Calcium

Vitamin D is the most significant nutrient in the absorption of calcium. Calcium absorption occurs through active vitamin D-dependent transport and through passive paracellular absorption that occur in the gut even though the vitamin D-dependent calcium binding protein calbindin D9k is also found in rat caecum and the large intestine [106]. A contributing factor to the development of osteoporosis is the lower plasma level of $1,25(OH)_2D$ (calcitriol). This is caused by a defect in the renal 1α-hydroxylase and an impaired conversion into a bioactive form of its molecule that leads to a decline in the vitamin D receptor creating relative resistance to absorption. This factor including inadequate exposure to sunlight and intake of diet rich in vitamin D contributes to hindered calcium absorption and leads to bone loss [123].

In addition to vitamin D, vitamins A and C can help support calcium transportation. However, high intake of either vitamin A or C can have a detrimental effect on bone health. Some dietary fat may also contribute to the process of gut absorption of calcium, however, high fat intake can interfere directly with gut calcium absorption [123]. Dietary protein argued to affect gut calcium absorption especially when taken outside the moderate proportion (approximate range of 1.0–1.5 g protein/kg are associated with normal calcium metabolism) [124]. Lactose is a recognised enhancer of gut absorption of calcium in mammals but debated in humans. Immediately lactose is hydrolysed into its constituent monosaccharides, glucose and galactose, lactose becomes an energy source for mammalian young as well as a nutrient suitable for the growth of the gut microbiota. In lactose-intolerant individuals, undigested lactose consumed in milk and/or dairy products without absorption in the small intestine arrive in the large intestine as a conditional prebiotic [125].

Studies in growing rats and humans have evaluated the effect of prebiotics on the gut microbiome while some were able to observe the effects on bone health. These studies are discussed in section 2.6.

2.7 Animal Studies on mineral absorption

Dietary galactooligosaccharide supplementation in rodents has been reported to have decrease caecal pH and increase caecal wall and content weight while faecal DNA quantitative PCR have also shown an increase in the proportion of *bifidobacteria* with galactooligosaccharide [106].

This study by Weaver et al. 2011 indicated that galactooligosaccharide treatment altered the gut microbiome, produced an increase in the abundance of *bifidobacteria*, and improved the calcium and magnesium absorption as well as bone strength and BMD in rats. From this 8-week feeding study of 0, 2, 4, 6 or 8% of prebiotic fiber, growing male Sprague-Dawley rats were used [126]. According to Weaver, they also evaluated at 4% eight prebiotic fibers for 12 weeks in a male rat model, the result showed elevated caecal SCFA and increase in mineral absorption as well as bone mineral status [106]. In another study when given difructose anhydride III (DFAIII, a non-digestible disaccharide), increased calcium and iron absorption was observed [127]. Treatment of healthy female OVX rats with polydextrose and inulin-based fibre for 4 weeks improved magnesium and calcium absorption and utilisation and increased the production of SCFA and the caecal weight [128].

2.8 Animal studies on the effects of prebiotics on bone health

Other studies have shown that galactooligosaccharide enhanced calcium absorption and prevented bone loss in ovariectomised female Wistar rats [129]. Similar result has also been found involving diet treatment with chicory inulin in male Wistar rats [130]. Under pathological conditions, gastrectomised male rats when treated with fructo-oligosaccharide, had reduced bone loss and increased calcium absorption [131], likewise fructo-oligosaccharide-fed male Wistar rats showed enhanced femoral bone volume as well as calcium and magnesium levels [132]. Furthermore, the treatment of healthy female mice with agave fructan and inulin for 6 weeks improved calcium and magnesium absorption, lowered mucosal pH and markedly reduced bone loss caused by ovariectomy by improving bone formation and conserving the trabecular structure [133]. Using 8 various types of prebiotic fibers, this study using a weanling rat model (Sprague-Dawley) found that inulin had the highest production of SCFAs and greatest femoral calcium uptake meanwhile the most effective fibers were the soluble fibers which increased whole body BMC and femoral BMD as well as the cortical area and thickness, and resistance to fracture [134]. Dietary inulin improve mineral (Ca, Zn and Cu) retention and was a beneficial effect on bone quality in broiler chickens [135].

2.9 Human Studies on calcium absorption and the gut microbiome

In a study of young adolescents, a combination of short- and long-chain inulin-type fructans was given for a period of 1 year as a daily food supplement, 47% greater increase in BMD was found in comparison to the control group [136]. The authors suggested that it could have been as a result of calcium absorption, although, an immune system response and gut microbiome composition may have played a role.

In another double-blind cross-over study of adolescent girls for three weeks periods in random order, the effect of galacto-oligosaccharides on calcium absorption using the faecal microbiota showed increased levels of beneficial faecal *bifidobacteria* dose-dependently. There was also an increase in the levels of calcium absorption which was however independent of dose given. It was therefore suggested and recommended that a daily consumption of 5g of GOS increases calcium absorption and gut microbiome specifically *bifidobacteria* [137].

An increase in calcium absorption was observed in male adolescents after consumption of 15 g oligofructose or sucrose (control treatment) daily over 3 main meals for 9 days [138]. Meanwhile, Griffin et al., following girls' treatment with 8 g/d placebo (sucrose), oligofructose or the mixture inulin+oligofructose for 3 weeks found no difference between their oligofructose and placebo group however calcium absorption was higher in the inulin+oligofructose mixture group compared to placebo [139].

Consumption of transgalactooligosaccharides (Elix'or) was found to stimulate Ca absorption in postmenopausal women [55 to 65 y (mean 62 y)] in a double-blind, randomised crossover study [140]. These studies imply that prebiotics modulate calcium absorption in the colon.

2.10 Human studies on the effects of prebiotics in bone health

A study reported data on the effects of soluble maize fibre on short-term calcium absorption in adolescent children of both sexes. The study involved two 3-week metabolic balance studies that tested 0 g/d (control) and 12 g/d (treatment) in a random order including it in a low-Ca diet (600 mg/d). An increased calcium absorption associated with gut microbiome changes was found in those with a low-calcium diet taking a soluble maize fiber and a greater proportion of the phylum *Bacteriodetes* compared with the control. However, there were no changes in bone turnover markers [141]. This could be as a result of insufficient time length of the intervention study.

In a double-blind, placebo controlled cross-over study, 6 weeks of treatment with 10g/d of oligofructose-enriched inulin increased calcium and magnesium absorption in postmenopausal women (72 years old (SD6.4) years) relative to the placebo treatment and this was followed by a decrease in bone resorption [142].

Kim et al. showed the effects of chicory fructan fiber supplementation on mineral absorption and serum bone markers by using a parallel, randomised, double-blind design in Korean postmenopausal women (mean age 60-61y). Their investigation involved administering 2 doses (breakfast and dinner) of 4g chicory fructan fiber or a placebo (maltodextrin/sucrose mixture) for 3 months. After 3 months they observed a lower level of serum alkaline phosphatase (a marker for bone formation) in the fructan group than in the controls indicating a slower bone turnover. They also observed an increased calcium absorption without any urinary calcium loss in this fructan group [143]. However, there was no effect on BMD; this could have been as a result of the length of exposure.

The effect of galacto-oligosaccharides on calcium absorption and, importantly, the faecal microbiota has been examined in adolescent girls. Levels of beneficial faecal *bifidobacteria* were increased in a dose-dependent manner. Calcium absorption also increased, but this was independent of dose [137]. The same group showed microbiota changes accompanying increased calcium absorption in a low-calcium diet in adolescent children of both sexes taking a soluble maize fiber compared with control but no changes in markers of bone turnover [141].

Of important note is the fact that resistant starches and sugar alcohols increases the rate of mineral absorption and bone mineral content in the gastrointestinal tract [121]. In adult rats receiving inulin or resistant starch, absorption of calcium and magnesium was stimulated/increased. However, when a combination of the 2 treatments was given the effect on calcium absorption was more significant [144].

As can be noted from these studies above, prebiotics, probiotics and/or functional foods could benefit from longer treatment duration and treatment administration during early, growth or perimenopausal phase. A potentially promising result for the maintenance and improvement of bone health could result from treatment of subjects with the dietary combination of prebiotics and/or probiotics with isoflavones. Variations however may occur due to dosage differences, treatment length, age and background of subjects and gender. In addition, these dietary interventions may help with the regulation of immune cells by reduction in the circulatory pro-inflammatory cytokines in the body.

2.11 Inflammatory cytokines and bone health

Cytokines are complicated to study due to their synergistic effects and their ability to affect or enhance each other's secretion, for example, IL-1 and TNF-α [145]. However, the cytokine network is significant in the regulation of the immune cells (primarily lymphocytes and macrophages) and skeletal system where a natural balance is needed for the physiological and pathophysiological bone metabolic homeostasis.

Cytokines are also known as crucial regulators of the adipose tissue metabolism. Cell types, pre-adipocytes and mature adipocytes are able to promote/enhance secretion/production of cytokines and chemokines by mRNA expression notably in obese individuals [146, 147].

2.11.1 Inflammatory (osteoclastogenic) cytokines

Inflammatory cytokines are generally known for their catabolic effects on tissue metabolism and homeostasis as well as the intracellular actions and signalling pathway to osteoclastic differentiation [148]. More so, in postmenopausal women especially, with the coupling effect of oestrogen deficiency; elevated levels of inflammatory cytokines have been linked with lower BMD [145], and these have been termed 'inflammageing' [149]. Some studies, although limited and contradictory, have investigated the impact of these inflammatory cytokines on bone loss in postmenopausal women [150-154].

IL-1β is one of the interleukin-1 family that is known to stimulate receptor activator of nuclear factor κ B (RANKL) expression and osteoclastogenetic differentiation and it is produced by macrophages. There are two ways by which IL-1β acts – directly in the presence of mitogen-activated protein kinases (p38-MAPK) and RANKL levels and - indirectly promoting TNF-α by RANKL expression in stimulating osteoclastogenetic differentiation. IL-1 generally increases the production of RANKL in stromal cells. IL-1 acts in synergy with TNF-α, IL-6 and TNF-α and prostaglandin E2 (PGE2). Furthermore, studies have indicated IL-1β is a strong stimulator of OC differentiation and bone resorption via RANKL expression [155, 156]. Consequently, anti-IL-1 therapies have been used to treat patients in order to block the actions of IL-1 for example, IL-1 receptor antagonist (anakinra) and IL-1 blocker (canakinumab and rilonacept) [155].

TNF-α also known as cachexin or cachectin are cell signalling proteins produced by a variety of immune cells; mainly by macrophages as well as NK cells, mast cells, B and T (Th1) lymphocytes. TNF-α induces RANKL and RANK expression thereby stimulating osteoclastic differentiation [157]. TNF-α stimulates osteoclast activity and development therefore affecting bone metabolism. Anti-TNF-α has been used to combat various bone diseases and inflammatory arthropathies, for example, osteoarthritis and Crohn's disease [158].

IFN-α, a type I IFN will normally inhibit RANKL-induced osteoclastogenesis by decreasing c-Fos expression [159]. However, during certain bacterial and/or viral infections, increased production of inflammatory cytokines and inflammasome activation may be induced indirectly [155, 160]. IFN-α has not been well-researched.

IFN-γ is a type II IFN mainly produced by natural killer (NK) cells, NKT cells, cytotoxic T cells and Th1 cells. IFN-γ, although classified as an anti-osteoclastogenic cytokine due to its role in the inhibition of RANK-mediated signalling as a result of the induction of ubiquitin-dependent TRAF6 degradation *in vitro.* However, bone loss is enhanced by IFN-γ during pathophysiological conditions such as injury, infection, inflammation and oestrogen deficiency [161]. Antigen-driven T cells activation produces IFN-γ to indirectly enhance osteoclastogenesis by inducing RANKL and TNF-α expression in the activated T cells [161]. Though, this activity depends on the levels of osteoclastogenic cytokines present in the local environment [155]. Therefore, IFN-γ can be considered as possessing a dual role in osteoclastogenesis. More research is however needed for more definitive and conclusive knowledge of the procedure.

IL-6 is a pleiotropic cytokine that converts and sends signals through its receptor, consisting of an α-chain and a gp130 subunit [155]. Although, IL-6 may have both inflammatory and anti-inflammatory effects, in osteoporosis it has been found to act mainly as both an inflammatory and osteoclastogenic cytokine in bone health.

Studies have shown that elevated levels of IL-6 in osteoporosis upregulates RANKL thereby supporting osteoclastogenic indirectly through mesenchymal cells interactions [162, 163]. Paradoxically, the direct effects of IL-6 on the bone can have an inhibitory effect on osteoclastogenesis rather than stimulating effect in the upregulation of the OPG system [164, 165]. Furthermore, a natural synergy is proven to exist with TNF-α, IL-1 and TNF-α as well as PGE2 [157].

IL-8 has been reported to be responsible for the increase in RANKL-mediated NFATc1 activation as a potential stimulator of osteoclast differentiation and bone degradation [155]. Though, evidence of an opposing effect was found with an increasing release of nitric oxide [157], IL-8 is still being referred to as an autocrine regulatory factor for osteoclastogenesis.

There are six distinctive variant of IL-17 cytokines; IL-17A – F, produced by a lineage of T helper lymphocytes [166]. It induces the expression RANKL, IL-1, TNF-α, IL-6 and IL-8 expression and is a strong and potent stimulator of osteoclastogenesis. It has been reported to be responsible for loss of RANKL/OPG system balance leading to osteoclastogenesis directly. Reports however, has been made on its indirect action on inhibition of osteoclastogenesis by the induction of GM-CSF production is osteoblasts [167].

In addition, monocyte chemoattractant protein (MCP-1), also known as (C-C motif) ligand 2 (CCL2) are chemokines (or small cytokines) induced by the inflammatory response. They have been reported to play significant role in osteoclastogenesis [168]. MCP-1 enhances OC precursors' proliferation by the induction of active ERK and are induced by RANKL stimulation [169].

2.11.2 Anti-Inflammatory (anti-osteoclastogenic) cytokines

Most often anti-inflammatory cytokine exerts opposite effect of inflammatory cytokine on bone. However, cytokines work interactively in a spatial and dynamic network to create a balance of both the inhibitory and stimulatory effects [148].

IL-10 is known as a potent suppressor of osteoclastogenesis and an anti-inflammatory cytokine. It is a Th2 cytokine responsible for the upregulation of the expression of OPG and downregulation of the expression of RANKL, M-CSF and cytokines such as IL-1, IL-6 and TNF-a [170]. IL-10 also inhibits the expression of NFATc1 and it nuclear translocation [171].

IL-18 has been reported as anti-osteoclastogenic cytokine. IL-18 are mainly produced by macrophages, osteoblasts and Kupffer cells. IL-18 induces osteoclast apoptosis by increasing production of nitric oxide (NO) [157]. It also increases OPG secretion from stromal cells, GM-CSF from T-cells and increases release of IFN-y from T cells. However, there is also a claim that IL-18 increases RANKL production from T cells [172].

IL-33 has been classified as an anti-osteoclastogenic cytokine responsible for the induction of osteoclasts' apoptosis by the multiplication of proapoptotic molecules such as Fas, FasL and BAX. IL-33 inhibits RANKL-induced osteoclastogenesis by regulating the expression of BLIMP1 and interferon regulatory factor 8 [155, 157]. It has also been reported that IL-33 promotes IL-10 production [173]. Similarly, IL-33 has not been well-researched, therefore more studies are needed in this area.

2.11.3 Dual-role cytokines

Cytokines provide a dual-role in the regulation of the immune system. The following cytokines are considered as having a dual-role effect.

IL-12 has been reported as an osteoclastogenic and anti-osteoclastogenic cytokine. IL-12 is produced by monocytes, dendritic cells and macrophages. IL-12 induces osteoclast apoptosis by inhibiting RANKL-induced osteoclastogenesis [155], and increasing the secretion of IL-1b and Th1 derived cytokines such as IFN-y, IL-2 and TNF-α. It also reduces the rate of osteoclastogenesis by its indirect action via T cells and direct effects on osteoclasts [157]. IL-12 has also been implicated in a synergistical relationship with IFN-y, a dual-role cytokine and IL-18, a reported anti-osteoclastogenic cytokine [174].

IL-12p70 is one of the IL-12 family that is involved in the generation of Th1 T cells and is responsible for innate and adaptive immunity. The IL-12 family are characterised by the induction of IFN-γ [174]. Meanwhile, IL-23 and IL-27 have been reported as members of IL-12 family cytokines produced by dendritic cells [174]. Now, IL-23 is known to promote OC differentiation, proliferation and bone resorption [175, 176], while IL-17 is produced in response to the stimulation of IL-23 and is also a potent stimulator of osteoclastogenesis by the expression of RANKL. Lubberts et al. observed a reduction in the destruction of bone and cartilage in arthritis-induced IL-17 deficient mice [167]. This suggests a relationship between elevated levels of IL-12p70, IL-17, and IL-23 and bone degradation which could be as a result of increased bone remodeling.

IL-23 is generated by the macrophages and the dendritic cells and is responsible for bone degradation in osteoporosis, osteoarthritis and rheumatoid arthritis [157]. IL-23 increases IL-17, Th subpopulation expressing IL-17 (Th-17) and RANKL production in its osteoclastogenic mechanism of action [175, 176]. However, it also possesses anti-osteoclastogenic features such as its involvement in the reduction of the formation of osteoclasts via T cells and reduced fusion of osteoclasts precursors. IL-23 synergistically blocks osteoclastogenesis with IL-18 [155].

Although inflammation is an important way of the immune system's response to infections and injury, the effects of pro-inflammatory cytokines on bone needs to be regulated. Another seemingly important factor is the effect of gut-derived serotonin on bone health. There are conflicting results of the importance of this bioamine and neurotransmitter on physiological bone remodelling. These are discussed in section 2.12.

2.12 The effect of gut-derived serotonin on bone health

One of the most intensively studied research on the mechanism of bone remodelling regulators is the LDL receptor-related protein 5 (LRP5). Serotonin (5-hydroxytryptophan [5-HT]) was identified in 1948 as a molecule present in serum (sero) and able to affect vascular tone (tonin) [177]. Serotonin synthesis is a two-step process from the essential amino acid, L-tryptophan, which is first hydroxylated in a rate-limiting fashion into L-5-hydroxytryptophan by tryptophan hydroxylase (Tph) thereafter decarboxylated by an L–amino acid decarboxylase [178]. Serotonin, a critical regulator of bone mass achieves different tasks depending on its site of synthesis in the body. The brain-derived serotonin promotes osteoblast proliferation, while gut-derived serotonin suppresses it [179].

Growing and emerging evidences suggest that 5-HT has peripheral effects that go beyond the gut and cardiovascular system, including the skeleton. Bone cells need access to 5-HT, which osteoblasts, osteocytes and osteoclasts express as *Tph1* however, investigations by Yadav et al. [180] have indicated that 5-HT obtained from the gut and transported by the cardiovascular system is the main source of the skeletal 5-HT. Stimulation of 5-HT receptors which have been recognised in all major bone cell types (i.e. osteoblasts, osteocytes and osteoclasts) influences bone cell activities.

The effects of the skeletal component of 5-HT were found to involve the osteoblastic 5-HT_{1B} receptor, with activation of this receptor leading to a decrease in bone formation through a reduced osteoblast proliferation [181]. In osteoblasts, three 5-HT receptors were found to be expressed—5-$HT_{1B,\ 2A,\ and\ 2B}$. Some have exhibited the existence and functionality of 5-HT signaling in osteoclastic- and osteocytic-like cells , as well as the presence of 5-HT receptors other than 5-HT_{1B} in osteoblasts [178]. This mechanism by which 5-HT influences the skeletal system has both anabolic and catabolic skeletal effects. The prevailing effect therefore is being determined by the extracellular concentration of 5-HT and the respective 5-HT receptor/s activated [178].

5-HT involved in the pathophysiology of depression, and therefore studies of depression and antidepressant treatments (modulating the serotonin system) are relevant regarding bone outcomes [181]. Preclinical evidence demonstrates negative effects of altered 5-HT signaling on bone health [181, 182]. Especially, in the clinical use of selective serotonin reuptake inhibitors, a 5-HT transporter which has been shown to be associated with increased postmenopausal bone loss [178].

The impact of gut-derived serotonin on osteoblasts can be lessened, in principle, by two ways. 1) Targeting the synthesis or 2) Targeting the signalling mechanism through the receptor inactivation. The latter which is often used clinically has been shown to increase alcohol intake and aggression with general adverse effects [183].

Although some work has been done, the regulatory mechanism of LRP5 on bone mass has not been fully delineated. However, these studies interminably point towards the functional link between LRP5 and gut-derived serotonin suggesting that blood serotonin is a negative regulator of bone mass. Studies thereby propose that inhibition of its synthesis in the gut of humans can pose a novel target/approach to osteoporosis treatment: Further clinical and experimental studies using different models need to be carried out to elucidate the relationship between gut-derived serotonin and bone health.

2.13 Conclusion

In conclusion to the literature review part of this thesis, although studies have researched the relationship between diet and bone health, only a few has been able to integrate the impact of gut microbiome into these relationships. Meanwhile, no study has incorporated the role of an array of inflammatory cytokines on the bone health status in postmenopausal women. This has led to the current research study aims and questions which contributes to the investigation on the influence of the gut microbiome and inflammation on bone health status of postmenopausal women. The use of HiSeq™ Sequencing Systems for shotgun metagenomics is also novel. Prior to the present study, no research has previously used this technique; the few available studies have used 16S rDNA sequencing.

2.14 References

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Bolaji Lilian Ilesanmi-Oyelere
Name/title of Primary Supervisor:	Professor Marlena C. Kruger
Name of Research Output and full reference:	
Ilesanmi-Oyelere, B.L., Roy, N.C., Cook, J., Kruger, M.C. Associations between Self-Reported Physical Activity, Heel Ultrasound Parameters and Bone Health Measures in Post-Menopausal Women. Int. J. Environ. Res. Public Health 2019	
In which Chapter is the Manuscript /Published work:	Chapter 3
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GRS Version 4– January 2019

Chapter 3 Associations between Self-Reported Physical Activity, Heel Ultrasound Parameters and Bone Health Measures in Post-Menopausal Women

The objective was to determine the associations between data obtained from self-reported physical activity, heel ultrasound measures and bone health (spine, hip and femoral neck T-scores and whole-body BMD). This chapter presented in this thesis has been altered from the published journal article to include the sample size plot.

The chapter is published as:

Ilesanmi-Oyelere, B.L.; Roy, N.C.; Coad, J.; Kruger, M.C. Associations between Self-Reported Physical Activity, Heel Ultrasound Parameters and Bone Health Measures in Post-Menopausal Women. *Int. J. Environ. Res. Public Health* 2019, 16, 3177.

3.1 Abstract

Physical activity plays an important role in the maintenance of bone health from childhood through adulthood. This study aimed to explore the associations between self-reported physical activity (PA), activity energy expenditure (AEE), heel ultrasound parameters and bone health measures among older adult women. The AEE was estimated from the responses of questionnaires for 125 older adult women aged 54–81 years. The bone parameters were measured by dual energy x-ray absorptiometry (DXA) and heel ultrasound parameters by the heel quantitative ultrasound (QUS). This study showed that AEE and the metabolic equivalent task (MET) were positively correlated with the bone and heel ultrasound parameters. However, fat mass (FM) and fat percentage were negatively correlated with AEE and MET. In addition, the regression analysis showed that higher AEE was a strong predictor of a higher spine T-score ($\beta = 0.212$, $p = 0.015$), QUS T-score ($\beta = 0.239$, $p = 0.011$) and stiffness index ($\beta = 0.240$, $p = 0.010$) after adjusting for age, fat mass, lean mass, height and calcium intake. These results contribute to our understanding of the importance of physical activity in postmenopausal women by reiterating the benefits of physical activity for older adult women. Physical activity is an important tool for the prevention and management of osteoporosis.

3.2 Introduction

Globally, 61% of osteoporotic fractures occur in women. In addition, one in three women over the age of 50 worldwide experiences osteoporotic fracture [1]. The global burden of osteoporosis is increasing and its impact on the health system, economies and society will worsen [1]. Physical activity is a factor that can be modulated to reduce the risk of developing osteoporosis.

Physical activity is defined by the World Health Organization (WHO) as any bodily movement of the skeletal muscle which requires energy expenditure [2]. The composition and function of the human skeletal system is enhanced by physical activity, however this also deteriorates with age [3]. In addition, a lack of exercise and physical activity has been linked to bone loss and ultimately osteoporosis [4], the presence of which may cause falls that result in osteoporotic fractures in the elderly women.

Bone loss in women is accelerated with the onset of menopause, making this group the focus of attention worldwide in regard to osteoporosis. Regardless of the underlying factors in the aetiology of osteoporosis and bone loss, an adequate and appropriate exercise regime may be beneficial for the reduction or alleviation of bone loss in osteoporosis, thereby reducing the prevalence and incidence of fractures [5].

Studies have compared the efficiency and effectiveness of the quantitative ultrasound sonometry (QUS) and dual energy X-ray absorptiometry (DXA) in the measurement and prediction of bone status [6, 7]. However, DXA is still widely known to be the gold standard for bone mineral density (BMD) measurement and evaluation [7]. In addition, age, race, sex, hormones and heredity as well as environmental features, such as nutrition and physical activity, have been reported as important determining factors for bone health [4, 8]. Of these, nutrition and physical activity are the factors that can be easily be improved for bone health in a lifetime.

Furthermore, previous studies, including Cochrane reviews, have presented the effects of physical activity on bone strength across the lifespan [9, 10], in children and adolescents [11] as well as in women, especially for postmenopausal women [12-14]. A study from Italy on the effectiveness of physical activity types on BMD in osteoporotic patients reported progressive resistance strength training of the lower limbs to be most effective for the neck of the femur, while a multicomponent training programme was suggested for spine BMD intervention [15].

Neilson et al. defined activity energy expenditure (AEE) as a modifiable component of total energy expenditure (TEE) derived from both volitional and non-volitional activities [16]. TEE comprises multiple components, including physical activity energy expenditure (PAEE), resting energy expenditure (REE), and the thermic effect of food (TEF) [17]. This study estimated AEE from the metabolic equivalent task (MET). The MET is used to express the intensity and energy expenditure of activities among people of varying weight. Furthermore, AEE (measured in kilocalories or kilojoules) during a particular activity depends on the individual's total body mass. The energy cost of the same type of activity is different for individuals of different weight [18].

The impact of physical activity has been used to alleviate several obesity-related diseases and the overall burden of diseases in men and women alike [19]. Although studies have investigated the effects of long-term physical activity, or a physically active lifestyle, on adult bone health parameters, to the authors' knowledge, no study has reported on the relationship between recent short-term physical activity and measures of bone health. A specific tool to achieve this is the interviewer-administered New Zealand Physical Activity Questionnaire - short form (NZPAQ-SF).

The specific aim of this research was to investigate the relationship between self-reported AEE, MET and QUS heel ultrasound and DXA outputs in a cohort of postmenopausal women. It was hypothesised that the participants with higher reported physical activity (PA) measured by AEE and MET have higher BMD, that is, a positive association.

3.3 Materials and Methods

3.3.1 Study Design

A total of 125 postmenopausal women aged between 54 and 81 years participated in the "Bugs'n'Bones" study that took place in the Human Nutrition Research Unit of Massey University, Palmerston North campus from June to December 2017. The majority (n = 124, 99.2%) of the participants were New Zealanders of European descent, who self-reported their ethnicity as New Zealand Europeans or 'Pakeha'. The sample size of the cross-sectional study was calculated using G*Power software version 3.0.10 (Heinrich-Heine-Universität Düsseldorf, Düsseldorf, Germany) and eighty-eight subjects were required for a 95% power and an alpha of 5% for the T-test (Fig. 3-1). The outcome measure used was CTX-1. This study estimated a total of one hundred and fifty participants based on the osteoporosis incidence ratio of 3:1 in women. Two subjects were excluded from the study, one due to a ketogenic diet and the other due to health conditions.

The subjects were recruited by advertisement on campus, the Whanganui Chronicle and by using a recruitment agency; Trial Facts (https://trialfacts.com/). The inclusion criteria were confirmed as menopause of at least 5 years based on no menstruation. The exclusion criteria were the presence of any systemic disease, food intolerances which affect the gut, smoking and high intake of alcohol. The subjects with significant weight loss or weight gain within the past year were also excluded. All subjects were free living and apparently healthy. Written informed consent was obtained from the subjects before commencing data collection. This study was approved by the Massey University Human Ethics Committee: Southern A, Application 17/17. The study was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) with the number ACTRN12617000802303.

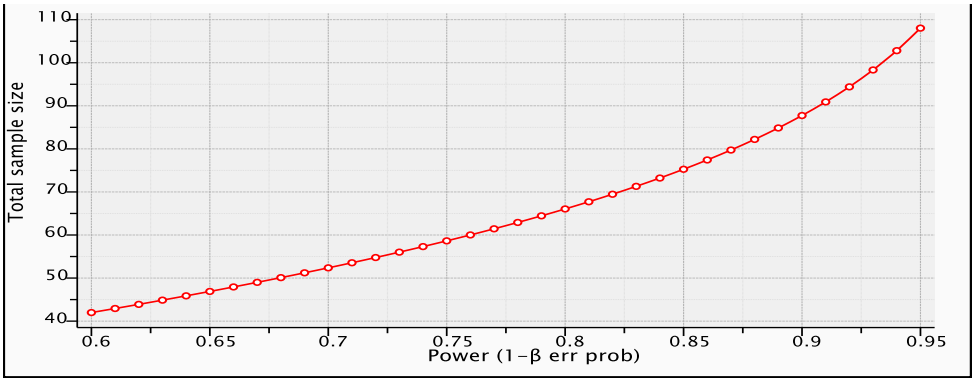

Figure 3-1 Sample size plot

T tests – Means: Difference between two independent means (two groups)

Tail(s) = Two, Allocation ratio N2/N1 =1, α err prob = 0.05, Effect size d = 0.7

3.3.2. Anthropometric Measurements of the Subjects

The body weight of participants was measured using the Detecto 437 eye-level weigh beam physician scale to the nearest 0.1 kg and standing height was measured using a wall mounted rolled stadiometer to the nearest 0.1 cm wearing light clothes and no shoes on. Body mass index (BMI) was calculated as the weight divided by height squared (kg/m^2). The waist to hip ratio was determined by measuring the waist and hip circumference to the nearest 0.1 cm using a non-stretchable measuring tape. The waist to hip ratio was used as a marker of abdominal obesity.

3.3.3. Physical Activity Questionnaire

The significance of physical activity was investigated amongst postmenopausal women in the Manawatu-Wanganui region of New Zealand [20] using the NZPAQ-SF to support and substantiate our previous article's findings of the same study. The previous study investigated lean body mass in relation to bone health [20]. More in-depth analyses of the data collected from the larger BugsnBones study were performed.

Physical activity was assessed using the NZPAQ-SF [21], which has previously been validated by Boon and colleagues [22]. Physical activities were quantified by MET-minutes/day and AEE-Cal/day which was calculated by using the scoring protocol of the international physical activity questionnaire (IPAQ) for continuous scores. The MET values and the formula for computation of MET-minutes were as follows [23].

Walking MET-minutes/week at work = 3.3 * walking minutes * walking days at work.

Moderate MET-minutes/week at work= 4.0 * moderate-intensity activity minutes * moderate intensity days at work.

Vigorous MET-minutes/week at work = 8.0 * vigorous-intensity activity minutes * vigorous intensity days at work Total Work MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores at work.

Total PA MET-minutes/week = sum of walking + moderate + vigorous MET-minutes/week scores.

The (NZPAQ-MVPA) was derived from the total weekly minutes of vigorous + moderate activity in bouts of ≥10 min excluding walking.

3.3.4. DXA and QUS Measurements

All body composition measurements, fat mass (FM), lean mass (LM) and fat percentage were measured and analysed using the Hologic QDR series Discovery A, Bone densitometry DXA, USA. The participants' BMD was measured at the femoral neck (FN), lumbar spine (LS) [L1–L4], trochanter, Ward's triangle and total hip. The DXA machine was calibrated every morning and at the end of each day for all measurements. The *in vivo* reproducibility of the coefficient of variation ranged between 0.34–0.70% for all measured sites. The reported LS BMD values were calculated as the means of four measured values from L1–L4. The Apex System Software version 4.5.3 (Hologic Inc., Bedford, MA, USA) was used for analysing the DXA scans. Osteoporosis was defined as a T score ≤ -2.5 and osteopenia as a T score between −1.0 and −2.5, according to the WHO criteria [24].

The Quantitative ultrasound (QUS) of the non-dominant heel was scanned using the GE Lunar Achilles II Portable Bone Densitometer (Hologic Inc., Northborough, MA, USA). The outputs included the stiffness index, the broadband ultrasound attenuation (BUA measured in dB/MHz), the speed of sound (SOS measured in m/s), the QUS T-score and QUS Z-score.

3.3.5. Calcium Intake Assessment

The participants' calcium intake assessment was investigated with a food frequency questionnaire (FFQ). The 108-item FFQ was used to collect information on the frequency of the participants' food and beverage intake utilising household measures for servings. The Food works version 9 Xyris software was used to analyse the participants' calcium intake data.

3.3.6. Statistical Analysis

IBM SPSS version 25 (IBM Company, Armonk, NY, USA) was used for all statistical analyses. The values of all variables' regional sites were presented as the mean ± standard deviation. The analysis of covariance (ANCOVA) was used to evaluate the differences in physical activity measures according to the hip osteoporotic groups (osteopenic and osteoporotic women) versus healthy women. The partial correlation analyses of the regional sites and QUS parameters with AEE and MET were performed and were adjusted for age, weight and height. The regression analysis of QUS parameters and skeletal site measurements by AEE was conducted and confounders such as age, fat mass, lean mass, height and dietary calcium intake were regressed into the model. The outcome variables used were the T-scores of skeletal sites as well as body composition measures. The regression analysis was used to obtain the determinants and predictors for the outcome variables, showing the relationship between these variables and bone health indicators. All *p*-values were reported significant at 0.05 or less.

3.4 Results

As shown in Table 3-1, the average weight of the subjects was 69.3 kg with a standard deviation of 11.2 kg. The average AEE calculated was 479 Cal/day from the physical activities recorded.

Table 3-1 The subjects' baseline characteristics and anthropometric variables.

Parameters	**(*n* = 125)**
	Mean ± SD
Age (years)	62.6 ± 4.5
Weight (kg)	69.3 ± 11.2
Height (cm)	162.3 ± 5.3
BMI (kg/m^2)	26.3 ± 4.2
WC (cm)	80.8 ± 10.8
HC (cm)	99.3 ± 7.6
WH ratio	0.8 ± 0.1
Spine BMC (g)	54.2 ± 11.4
Spine BMD (g/cm^2)	0.94 ± 0.15
Spine T-score	−0.9 ± 1.4
Femoral neck BMC (g)	3.6 ± 0.5
Femoral neck BMD (g/cm^2)	0.7 ± 0.1
Femoral neck T-score	−1.2 ± 0.9
Hip BMC (g)	29.9 ± 5.1
Hip BMD (g/cm^2)	0.9 ± 0.1
Hip T-score	−0.7 ± 1.0
Whole body total fat mass (kg)	29.4 ± 8.3
Whole body total lean mass (kg)	40.6 ± 4.5
Whole body total fat %	41.2 ± 6.5
Stiffness index	88.9 ± 13.6
BUA (dB/MHz)	110.7 ± 11.2
SOS (m/s)	1554.4 ± 31.0
QUS T-score	−0.7 ± 0.9
QUS Z-score	0.8 ± 0.8
AEE (Cal/day)	479.1 ± 772.7
MVPA MET-minutes/week	1644.0 ± 1970.4
Walk MET-minutes/week	624.8 ± 839.1
Moderate MET-minutes/week	918.2 ± 1028.3
Vigorous MET-minutes/week	725.8 ± 1414.4
Total MET-minutes/week	2268.8 ± 2374.5
Calcium intake (mg/day)	1263.5 ± 851.4

SD = standard deviation; BMI = body mass index; WC = waist circumference; HC = hip circumference; WH = waist to hip; BMC = bone mineral content; BMD = bone mineral density; BUA = broadband ultrasound attenuation; SOS = speed of sound; QUS = Quantitative Ultrasound; AEE = activity energy expenditure; MET = metabolic equivalent of task.

The classification into osteoporotic status based on the hip revealed that healthy individuals were significantly different than the osteoporotic groups (F_{2119} = 5.77, p = 0.004). The significant differences were also observed between the groups for age and weight according to the hip osteoporotic status (F_{1119} = 5.97, p = 0.016) and (F_{1119} = 33.01, $p \leq 0.001$) respectively but not for height (Table 3-2).

Table 3-2 Summary of ANCOVA according to hip osteoporotic status.

ANCOVA Based on the Hip Osteoporotic Status					
Parameters	**Sum of Squares** [1]	**Degrees of Freedom**	**Mean Square**	**F**	***p*-Value**
Tertiles of Total METs	2.033	2	1.017	5.769	0.004
Age (years)	1.052	1	1.052	5.967	0.016
Weight (kg)	5.818	1	5.818	33.013	<0.001
Height (cm)	0.058	1	0.058	0.327	0.568
Error	20.972	119	0.176		
Total	50.000	125			

[1] R Squared = 0.301.

Taking into account the effects of age, weight and height, the partial correlation coefficients are as shown below. The effects of AEE and MET were only significant for femoral neck BMD and the T-score. Similarly, the relationship between the stiffness index and measures of PA measures were significant. The positive correlations were observed between LM and AEE (r = 0.140, p = 0.130) and MET (r =0.177, p = 0.056) were not significant. However, there were negative correlations between AEE and fat percentage (r = −0.160, p = 0.083), likewise MET and fat percentage (r = −0.229, p = 0.013). The strongest correlations were found between the PA measures and the QUS parameters (Table 3-3).

Table 3-3 The partial correlation coefficients of bone health and quantitative ultrasound (QUS) parameters with PA measures adjusted for age, weight and height.

Bone Health Parameters	Activity Energy Expenditure (Cal/day)	Metabolic Equivalent of Task (minutes/week)
Spine BMD (g/cm^2)	0.110 ^{ns}	0.106 ^{ns}
Spine T-score	0.104 ^{ns}	0.100 ^{ns}
Femoral neck BMD (g/cm^2)	0.208 *	0.212 *
Femoral neck T-score	0.206 *	0.210 *
Hip BMD (g/cm^2)	0.144 ^{ns}	0.147 ^{ns}
Hip T-score	0.121 ^{ns}	0.123 ^{ns}
Stiffness Index	0.312 ***	0.321 ***
BUA (dB/MHz)	0.162 ^{ns}	0.160 ^{ns}
SOS (m/s)	0.341 ***	0.356 ***
QUS T-score	0.313 ***	0.323 ***
QUS Z-score	0.315 ***	0.323 ***
Fat mass (kg)	−0.134 ^{ns}	-0.170 ^{ns}
Lean mass (kg)	0.140 ^{ns}	0.177 ^{ns}
Fat %	−0.160 ^{ns}	-0.229 *

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$ (two tailed). BMD = bone mineral density; BUA = broadband ultrasound attenuation; SOS = speed of sound; QUS = Quantitative Ultrasound; ns = not significant.

To assess the extent of the impact of body composition, the calcium intake and physical activity on QUS and bone measures, the regression analysis was performed. Table 3-4 displays age, fat mass, lean mass, height, dietary calcium intake and AEE (Cal/day) as a predictor for bone health measures and QUS parameters. The dependent variables included were regional site T-scores, QUS outputs and body composition. The regression analysis showed that higher AEE was a strong predictor of higher spine T-score ($\beta = 0.212$, $p = 0.015$), QUS T-score ($\beta = 0.239$, $p = 0.011$) and the stiffness index ($\beta = 0.240$, $p = 0.010$) after adjusting for age, weight, height and calcium intake. However, AEE was not significantly associated with the hip or femoral neck T-scores.

As shown in Table 3-4, the regression analysis shows the relationship between the dependent variables; spine, hip, femoral neck and QUS T-scores, lean mass and fat mass, and AEE.

Table 3-4 Regression analysis of bone health, QUS parameters and body composition.

Parameters	**β**	**CI**	**R^2**	***P***
Spine T-score			0.341	
Age (years)	0.020	−0.043, 0.055		0.803
Fat mass (kg)	0.293	0.016, 0.079		0.003
Lean mass (kg)	0.292	0.019, 0.159		0.013
Height (cm)	−0.155	−0.092, 0.010		0.116
Calcium intake (mg/day)	0.223	0.0001, 0.001		0.006
AEE (Cal/day)	0.212	0.000,0.001		0.015
Femoral neck T-score			0.367	
Age (years)	−0.241	−0.079, −0.017		0.003
Fat mass (kg)	0.142	−0.006, 0.036		0.150
Lean mass (kg)	0.378	0.029, 0.118		0.001
Height (cm)	0.001	−0.033, 0.033		0.991
Calcium intake (mg/day)	0.068	0.000, 0.0002		0.397
AEE (Cal/day)	0.156	−0.000, 0.001		0.060
Hip T-score			0.338	
Age (years)	−0.226	−0.084, −0.015		0.006
Fat mass (kg)	0.254	0.007, 0.051		0.011
Lean mass (kg)	0.348	0.026, 0.124		0.003
Height (cm)	−0.037	−0.043, 0.029		0.710
Calcium intake (mg/day)	0.018	−0.0002, 0.0002		0.821
AEE (Cal/day)	0.015	−0.0002, 0.0002		0.863
Whole body BMD			0.198	
Age (years)	−0.133	−0.008, 0.001		0.136
Fat mass (kg)	0.034	−0.002, 0.003		0.754
Lean mass (kg)	0.295	0.001, 0.013		0.022
Height (cm)	0.025	−0.004, 0.005		0.815
Calcium intake (mg/day)	0.204	0.000, 0.000		0.022
AEE (Cal/day)	0.015	−0.000, 0.000		0.873
QUS T-score			0.245	
Age (years)	−0.197	−0.071, −0.005		0.024
Fat mass (kg)	0.026	−0.018, 0.024		0.805
Lean mass (kg)	0.312	0.013, 0.107		0.013
Height (cm)	−0.031	−0.040, 0.029		0.771
Calcium intake (mg/day)	−0.102	−0.000, 0.0001		0.234
AEE (Cal/day)	0.239	0.000, 0.001		0.011
Stiffness Index			0.246	
Age (years)	−0.196	−1.133, −0.083		0.024
Fat mass (kg)	0.022	−0.301, 0.373		0.832
Lean mass (kg)	0.314	0.214, 1.999		0.012
Height (cm)	−0.031	−0.633, 0.469		0.769
Calcium intake (mg/day)	−0.098	−0.004, 0.001		0.252
AEE (Cal/day)	0.240	0.001, 0.007		0.010

BMD = bone mineral density; QUS = Quantitative Ultrasound; AEE = activity energy expenditure; CI = 95% confidence interval. Model predictors are age, fat mass, lean mass, height, calcium intake and AEE.

3.5 Discussion

The study sought to determine the extent to which self-reported physical activity (calculated as AEE) predicts bone, QUS parameters and body composition for New Zealand European postmenopausal women. This work provided detailed anthropometry measurements, ANCOVA comparison of healthy and osteoporotic groups according to their self-reported physical activity as well as the partial correlation analyses for bone and physical activity parameters. Comparisons between the groups revealed higher AEE and MET for the healthy group than the osteoporotic group. Although not significant, the positive partial correlations were observed between the physical activity measures (calculated AEE and MET) and bone health parameters. The partial correlations between physical activity measures and the FM and fat percentage were negative.

Furthermore, the regression analysis showed the extent to which self-reported AEE may determine the bone health, QUS and body composition status in older women after adjusting for confounders. In keeping with other studies [4, 25], AEE was positively associated with the spine T-score, QUS T-score, and the stiffness index but no significant association was found with the femoral neck and hip T-scores in postmenopausal women. The results in this chapter indicate that higher AEE is strongly associated with higher spine T-scores and QUS parameters among older adult women.

The relationship between self-reported physical activity and bone health was more pronounced for the QUS of the heel which may be because the heel bears a higher load in comparison to other parts of the body. The partial correlations also revealed positive associations between the femoral neck and PA measures. This is especially important since fragility fractures mainly severely occur at the femoral neck of the hip. The data from this study showed two women reported no physical activity in the week prior to the study date. One was osteopenic and the other healthy, based on the spine classification.

Similar studies have been conducted by other researchers looking at the relationship between self-reported and measured/monitored AEE and bone health [8]. A study by Milliken et al. [26] investigated the effects of exercise on BMD in postmenopausal women. The form of physical activity used was a one-year supervised exercise training programme, measured as the total amount of weight lifted for the duration of the programme. The results of this study indicated a positive effect of exercise on trochanter and FN-BMD, but not that of LS. The authors found that the cumulative weight lifted predicted BMD of the trochanter [27]. Nevertheless, the present study differs from that of Milliken et al. (2006) due to the fact that there was not a relationship between FN-BMD and exercise. This may be as a result of the sample size. However, the lumbar spine BMD and the T-score was positively associated with AEE.

Body weight, age, sex and the duration of sleep influence AEE. Therefore, the individuals who perform the same amount of activity and weigh more, expend more energy than those who weigh less [28]. This study found negative correlations between FM, fat percentage and self-reported AEE. Furthermore, the result of this study is similar to previously reported papers which linked physical inactivity with body weight and obesity [19, 25, 29]. This study found physical inactivity was associated with a higher percentage of body fat and FM.

In summary, these results show that physical activity is associated with higher bone mass, the stiffness index, lean body mass and a lower percentage of fat and FM. The strengths of this research in terms of the self-reported NZPAQ include its low-cost methodology, the fact that recall does not alter behaviour and its suitability for the population being studied. The limitations of this research include its cross-sectional nature. Therefore, problems of causal associations exist, and the use of self-reported physical activity from the questionnaires for estimating AEE. However, the MET has been reported as an acceptable methodology for measuring physical activity [4]. This study needs to be replicated in other ethnicities and communities and comparison studies are warranted to be representative of the general population.

3.6 Conclusions

In conclusion, this study evaluated the self-reported AEE as a predictor of bone and QUS status, the stiffness index and body composition. AEE showed a strong positive association with bone health measures. This supports the aim of this research which shows that PA is positively related to bone health status (especially femoral neck BMD and T-score) in older adult women and negatively associated with fat percentage and FM. This study is the first to use the NZPAQ-SF to investigate the relationship between self-reported physical activity and bone health.

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MASSEY UNIVERSITY
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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Bolaji Lilian Ilesanmi-Oyelere
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Name of Research Output and full reference:	
Ilesanmi-Oyelere, B.L.; Coad, J.; Roy, N.; Kruger, M.C. Lean body mass in the prediction of bone mineral density in postmenopausal women. BioResearch Open Access 2018	
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Involved in the study design, participant recruitment, running of the study and data collection, analysis and interpretation and writing of the first manuscript draft	
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Date:	15 November 2019

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Chapter 4 Lean body mass in the prediction of bone mineral density in postmenopausal women

Based on the fact that physical activity has positive effects on bone health, the objective of this part of the study was to determine the associations between body composition obtained from DXA measurements, heel ultrasound measures and spine, hip, femoral neck and whole body BMD sites. This chapter explores the contribution of lean body mass also known as skeletal muscle mass to the determination of bone health status in postmenopausal women.

This chapter is published as:

Ilesanmi-Oyelere, B.L.; Coad, J.; Roy, N.C.; Kruger, M.C. Lean body mass in the prediction of bone mineral density in postmenopausal women. *BioResearch Open Access* 2018, 7:1, 150-158.

4.1 Abstract

Due to conflicting results of the association between body composition and bone mineral density (BMD), we investigated the relationship between fat mass (FM), lean mass (LM) and BMD in New Zealand postmenopausal women. We hypothesised that increased LM will indicate a higher BMD. A cross sectional study was performed examining the associations between body composition, anthropometric measures, activity energy expenditure (AEE) and bone health status [using dual-energy X-ray absorptiometry (DXA)]. One hundred and twenty-seven (127) healthy postmenopausal women aged between 54 – 81 years. Both FM and LM were significantly associated with spine, hip, femoral neck and whole body BMD. However, LM and not FM was the strongest predictor of femoral neck (FN) BMD ($\beta = 0.497$, $p<0.001$), hip BMD ($\beta = 0.495$, $p<0.001$), spine BMD ($\beta = 0.449$, $p<0.001$) and whole body BMD ($\beta = 0.406$, $p<0.001$). Age was negatively associated with FN and hip BMD. Lean mass was positively associated with FN, spine, hip and whole body BMD. The findings of this research suggest the need to increase LM rather than FM highlighting the importance of physical activity for this age group.

4.2 Introduction

Postmenopausal osteoporosis is a disease of public health concern and due to its debilitating nature affects the aged, especially elderly women. Osteoporosis in postmenopausal women is associated with a reduction in oestrogen levels, which consequently results in the acceleration of bone fragility and fracture [1]. The World Health Organization (WHO) defines osteoporosis as a disease that is characterised by low bone mass and micro-architectural deterioration of bone tissues, leading to bone fragility and increased fracture risk. The diagnosis of osteoporosis according to WHO may be obtained from one or more of the following regions: total hip, femoral neck (FN) and lumbar spine [2].

The disease state may result in fracture, which could subsequently lead to a lack of independence and mobility. Body composition is an important part in the determination of bone mineral density (BMD) and bone mineral content (BMC) as well as osteoporotic status. Body weight tends to have the capacity to elevate bone mineral status due to its ability to exact mechanical force and action on the host. Lean, fat, and bone mass are the three components of body weight found to be associated with bone status [3]. Lean mass, fat mass, bone mass and water together accounts for approximately 90-95% of the body weight [4].

Many epidemiological studies have reported and suggested that both FM and LM may affect bone mass status especially in the aged group [5, 6]. Adipose tissue is metabolically active therefore its effects on the bone or skeleton may be regulated by the weight-bearing effect as well as non-weight-bearing effects [5]. Examples of the non-weight-bearing effect include the hormonal metabolism of the adipocytes, such as leptin, insulin-like growth factor I and several cytokines. It has thus been reported to a degree of conclusion that weight-bearing and resistance-type physical activity has a positive effect and can serve as a measure for the prevention of osteoporosis [5, 7].

Studies have controversially reported on whether being overweight and obese results in a detrimental or protective effect on bone health. Both fat and bone cells originate from the same bone marrow stem cells [8, 9] and it is well known that physical inactivity and ageing induces both obesity and osteoporosis [10]. In addition, these two disorders synergistically induce functional impairments and physical disabilities, which suggest a complex effect of obesity on bone health. The protective effect of obesity on bone mass has therefore being termed 'obesity paradox' or 'reverse epidemiology' [5, 11].

The comparative contribution of the body fat and LM (or fat-free mass) to BMD variation has been controversial based on the original research findings available. Some studies [12-14] have reported that LM, not FM is associated with bone mass, while others [5, 15] have found that FM, not LM is important in the determination of BMD. Whereas, some have indicated that both FM and LM can equally serve as a predicting factor for BMD [16]. Furthermore, some studies have reported that LM is of more importance than FM in premenopausal women and FM more significant than LM in postmenopausal women [17, 18]. However, other studies has shown that LM was associated with BMD in both premenopausal and postmenopausal women [8, 19]. Furthermore, some studies made observations that FM was associated with BMD in men under 50 years, meanwhile, this was not the case in women and men over 50 years [5]. The inconsistency in the findings may be due to methodology and inadequately powered study design. In addition, Ho-Pharm et al. suggested that age, ethnic group and sex play a major role in the relative contribution of body composition parameters to BMD [6] as well as the site of measurement.

Owing to the presence of conflicting findings in the relationship between body composition and bone density, this study will shed light in terms of New Zealand postmenopausal women's perspective. Two research questions guided this study: 1) How are body composition measures such as, FM or LM related to regional and whole body measures such as femoral, hip, spine, and whole body BMD? 2) How do the regional and whole body measures relate to anthropometric variables such as weight and BMI as well as Quantitative Heel Ultrasound (QUS) T-score and the activity energy expenditure (AEE)?

4.3 Materials and Methods

4.3.1 Study design

A total of one hundred and twenty seven postmenopausal women aged between 54 and 81 years participated in the "Bugs'n'Bones" study that took place in the Human Nutrition Research Unit of Massey University, Palmerston North campus from June to December 2017. Sample size was calculated using G*Power software version 3.0.10 and eighty-eight subjects were required for a 90% power and an alpha of 5% for T-test. A total of one hundred and fifty was required based on osteoporosis incidence ratio of 3:1 in women. In this cross-sectional study, two subjects were excluded from the study, one due to a ketogenic diet and the other due to health conditions. Subjects were recruited by advertisement on campus, the Whanganui Chronicle and by using a recruitment agency; Trial Facts (https://trialfacts.com/). The inclusion criteria were confirmed as menopause of at least 5 years based on no menstruation. Exclusion criteria were the presence of any systemic disease, food intolerances which affects the gut, smokers and high intake of alcohol. Subjects with significant weight loss or weight gain within the past year were excluded. All participants completed the New Zealand Physical Activity Questionnaire (NZPAQ) [[20]] and the AEE was calculated. All subjects were free living and apparently healthy. Written informed consent was obtained from subjects before commencing data collection. The study was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) with the number ACTRN12617000802303. This study was also approved by Massey University Human Ethics Committee: Southern A, Application 17/17, following the Helsinki Declaration of 1975, as revised in 2008.

4.3.2 Anthropometric and body composition measurements of the subjects

Body weight of subjects was measured using the Detecto 437 eye-level weigh beam physician scale to the nearest 0.1kg and standing height was measured using a stadiometer to the nearest 0.1cm wearing light clothes and no shoes on. The BMI was calculated as weight divided by height squared (kg/m^2). Waist to hip ratio was determined by measuring the waist and hip circumference to the nearest 0.1cm using a non-stretchable measuring tape. Waist to hip ratio was calculated as a marker of abdominal obesity. Stiffness index, QUS T-score and Z-score of the non-dominant heel were scanned using the GE Lunar Achilles II Portable Bone Densitometer. The stiffness index is a composite ultrasonometry variable of two component variables of speed of sound (SOS) and broadband ultrasound attenuation (BUA) used to identify low BMD and/or osteoporotic fracture. This is based on the principle that bone as a porous material will absorb, scatter and transmit sound wave dependent on stiffness, density and volume [22,23].

Body composition measurements, FM, LM and fat percentage were measured and analysed using the Hologic QDR series Discovery A, Bone densitometry system [Dual energy X-ray Absorptiometry (DXA)]. BMD was measured at the FN, lumbar spine (LS) [L1-L4], trochanter, Ward's triangle and total hip. The DXA machine was calibrated every morning for all the measurements and at the end of each day. The *in vivo* reproducibility of the coefficient of variation ranged between 0.34-0.70% for all measured sites. The reported BMD values were calculated as means of four measured values from L1–L4. Apex System Software version 4.5.3 was used for analysing the DXA scans. Osteoporosis was defined as a T score ≤ 2·5 and osteopenia as T score between -1.0 and -2.5 according to the WHO criteria [2].

4.3.3 Statistical analysis

IBM SPSS version 25 (IBM Company, Armonk, NY, USA) was used for all statistical analyses. The outcome variables used were BMD of whole body and at skeletal sites. The values of all variables for the whole body and regional sites were presented as mean ± standard deviation. Comparisons of the mean values of two groups of healthy and osteopenic/osteoporotic subjects classified according to their spine T-scores were analysed by independent t-test as parametric variables. The mean difference of other groups of subjects with BMI<25kg/m^2 and BMI≥25kg/m^2 were compared using independent t-test. Correlation analyses of the whole body, regional sites BMD, and T-scores with the independent variables such as age, weight, BMI, AEE and QUS T-score were performed to obtain the Pearson's correlations. Stepwise multiple linear regression analysis was used to obtain the determinants/predictors for the outcome variables. All p-values were reported significant at 0.05 or less.

4.4 Results

Table 4-1 shows the demography, body composition and lifestyle characteristics of the 125 women studied. The BMI of the women ranged from 14.9 to 44.0 kg/m^2. According to the WHO classification, 2.4% of the women were underweight, 34.4% were of normal weight and 48% were overweight while 15.2% were obese.

Table 4-1 Subjects' baseline characteristics and anthropometric variables

Parameters	Mean ± SD (n=125)	Range	
		Min	Max
Age (years)	62.6±4.5	54.0	81.0
Weight (kg)	69.3±11.2	43.0	110.8
Height (cm)	162.3±5.3	149.1	175.4
BMI (kg/m^2)	26.3±4.2	14.9	44.0
WC (cm)	80.8±10.8	57.0	110.0
HC (cm)	99.3±7.6	78.0	122.5
Spine area (cm^2)	57.4±6.0	23.7	71.2
Spine BMD (g/cm^2)	0.94±0.15	0.5	1.3
Spine BMC (g)	54.2±11.4	26.7	82.6
Spine T-score	-0.9±1.4	-4.6	2.6
Femoral neck area	5.0±0.4	3.9	6.3
Femoral neck BMC (g)	3.6±0.5	2.4	5.2
Femoral neck BMD (g/cm^2)	0.71±0.10	0.5	1.0
Hip area (cm^2)	40.0±3.4	27.2	44.3
Hip BMC (g)	29.9±5.1	19.0	44.2
Hip BMD (g/cm^2)	0.85±0.11	0.6	1.2
Hip T-score	-0.7±1.0	-2.5	2.1
Whole body area (cm^2)	1952.6±143.2	1641.3	2387.6
Whole body BMC (g)	2207.0±333.7	1618.3	3385.5
Whole body BMD (g/cm^2)	1.13±0.11	0.9	1.5
Whole body Total Fat Mass (kg)	29.4±8.3	6.4	56.5
Whole body Total Lean Mass (kg)	40.6±4.5	30.7	57.3
Whole body Total Mass (kg)	70.0±11.2	43.1	112.6
Whole body Total %Fat	41.2±6.5	14.8	52.8
Stiffness index	88.9±13.6	54.0	137.0
QUS T-score	-0.7±0.9	-2.9	2.3
QUS Z-score	0.8±0.8	-1.2	4.0
AEE (Cal/day)	3056.10±10793.96	0.0	101207.2

SD=standard deviation; BMI=body mass index; WC=waist circumference; HC=hip circumference; QUS=Quantitative Ultrasound Sonometry; BMD=bone mineral density; BMC=bone mineral content; AEE=activity energy expenditure.

Fig. 4-1 to 4-3 shows the spine BMD, hip BMD and stiffness index with respect to the quartile distributions of LM. The BMD and stiffness index increased linearly with an increase in LM in all the three measures.

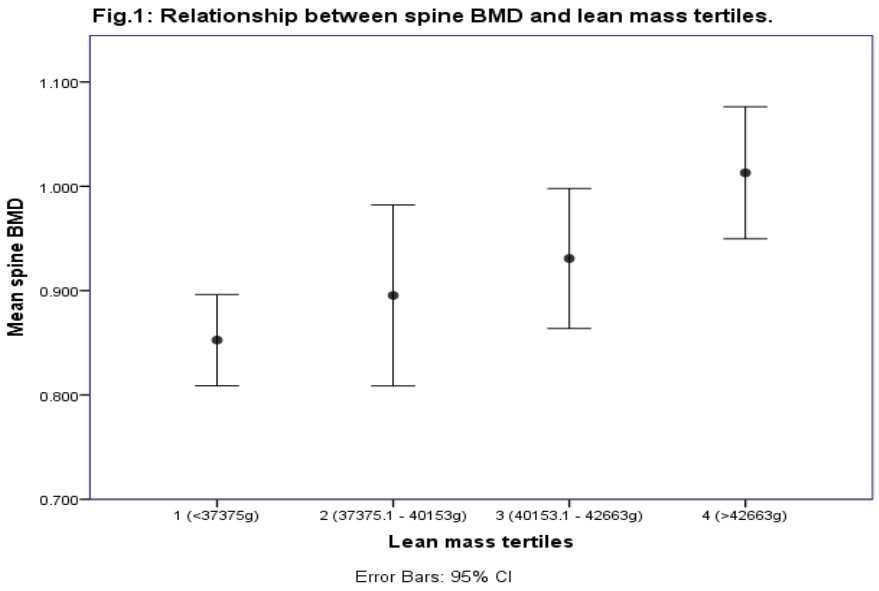

Figure 4-1 The relationship between spine BMD and lean mass tertiles.

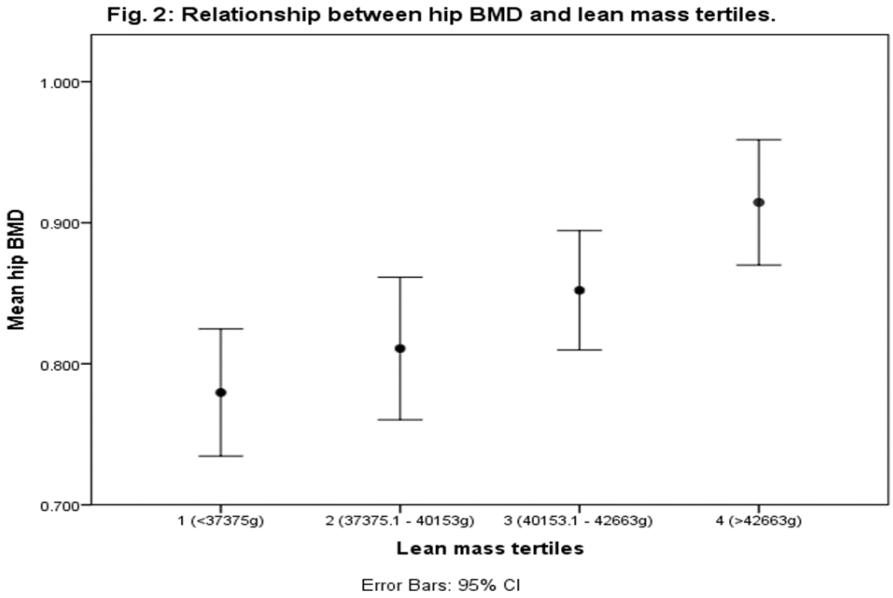

Figure 4-2 The relationship between hip BMD and lean mass tertiles.

Figure 4-3 The relationship between stiffness index and lean mass tertiles

In Table 4-2, to test the hypothesis that osteopenic/osteoporotic and women with normal bone mass have equal mean body compositions; an independent t-test was performed. In this selected population, based on the T-score, there were 60 women with normal bone mass (T-score ≥-1.0) and 65 women with osteopenia/osteoporosis (T-score < -1.0). The osteopenic/osteoporotic group of women were slightly older, shorter and thinner. They had lower BMC, BMD, bone area, body mass, body mass components and AEE than the healthy women (Table 4-2).

Osteopenic/osteoporotic women (M=62.9years, SD=4.0) and those with normal bone mass (M=62.3years, SD=5.0) did not differ significantly according to their age [$t(123) = -0.74$, $p = 0.463$]. Conversely, concerning FM and LM; osteopenic/osteoporotic women [(M=26.7kg, SD=7.6) and (M=38.8kg, SD=3.6)] and women with normal bone mass [(M=32.2kg, SD=8.2) and (M=42.6kg, SD=4.6)] were significantly different, $t(123) = 3.87$, $p < 0.001$ and $t(123) = 5.11$, $p < 0.001$ respectively.

Table 4-2 Comparison of subjects' anthropometric and DXA data without and with osteoporosis

Parameters	Women without osteoporosis (n=60)	Women with osteopenia/osteoporosis (n=65)	Independent *t*-test
	Mean±SD		*P*
Age (years)	62.3±5.0	62.9±4.0	0.463
Weight (kg)	74.0±10.9	64.9±9.8	<0.001
Height (cm)	163.2±5.9	161.5±4.6	0.084
BMI (kg/m^2)	27.9±4.3	24.9±3.6	<0.001
WC (cm)	84.7±10.9	77.2±9.5	<0.001
HC (cm)	101.7±6.8	97.2±7.8	0.001
Spine area (cm^2)	58.9±6.8	56.1±4.9	0.009
Spine BMD (g/cm^2)	1.07±0.10	0.82±0.08	<0.001
Spine BMC (g)	63.0±9.3	46.1±6.1	<0.001
Spine T-score	0.2±0.9	-2.0±0.7	<0.001
Femoral neck area	5.0±0.5	5.0±0.4	0.764
Femoral neck BMC (g)	3.8±0.5	3.3±0.4	<0.001
Femoral neck BMD (g/cm^2)	0.76±0.09	0.66±0.07	<0.001
Hip area (cm^2)	35.2±3.8	34.8±3.0	0.430
Hip BMC (g)	32.4±5.2	27.6±3.8	<0.001
Hip BMD (g/cm^2)	0.92±0.11	0.79±0.77	<0.001
Hip T-score	-0.2±0.9	-1.2±0.7	<0.001
Whole body area (cm^2)	2029.1±134.2	1881.9±112.0	<0.001
Whole body BMC (g)	2419.7±318.7	2010.7±201.4	<0.001
Whole body BMD (g/cm^2)	1.19±0.10	1.07±0.08	<0.001
Whole body Total Fat Mass (kg)	32.2±8.2	26.7±7.6	<0.001
Whole body Total Lean Mass (kg)	42.6±4.6	38.8±3.6	<0.001
TFM/TLM Ratio	0.8±0.2	0.7±0.2	0.031
Waist/Hip Ratio	0.8±0.1	0.8±0.1	0.009
Stiffness index	93.7±13.7	84.5±12.0	<0.001
QUS T-score	-0.4±0.9	-1.0±0.8	<0.001
QUS Z-score	1.0±0.8	0.5±0.8	<0.001
AEE (Cal/day)	4724.0±14380.7	1516.5±5483.9	0.097

SD=standard deviation; BMI=body mass index; WC=waist circumference; HC=hip circumference; QUS=Quantitative Ultrasound Sonometry; BMD=bone mineral density; BMC=bone mineral content; AEE=activity energy expenditure.

Similar to Table 4-2, a t-test was performed to observe the contribution of BMI to LM and FM. A total of 79 women with BMI ≥ 25kg/m^2 had significantly higher weight, BMI, WC, HC and BMC, BMD and T-score but lower AEE than those with a BMI < 25kg/m^2 (n=46) as can be observed in Table 4-3. Women with BMI ≥ 25kg/m^2 [FM (M=33.9kg, SD=6.2) and LM (42.0kg, SD=4.3)] had significantly higher body compositions than those with BMI < 25kg/m^2 [FM (M=21.5kg, SD=4.9) and LM (M=38.3kg, SD=4.0)]; FM, $t(123) = -11.50$, $p < 0.001$ and LM, $t(123) = -4.72$, $p < 0.001$ (Table 4-3).

Table 4-3 Comparison of anthropometric and DXA data of subjects with BMI<25kg/m² and BMI≥25kg/m²

Parameters	BMI<25kg/m² (n=46)	BMI≥25kg/m² (n=79)	Independent *t*-test
	Mean±SD		*P*
Age (years)	62.1±4.2	62.9±4.7	0.328
Weight (kg)	58.8±6.5	75.4±8.6	<0.001
Height (cm)	162.5±5.4	162.2±5.3	0.796
BMI (kg/m^2)	22.3±2.0	28.7±3.2	<0.001
WC (cm)	71.2±6.9	86.5±8.4	<0.001
HC (cm)	92.9±5.6	103.1±6.0	<0.001
Spine area (cm²)	57.7±5.6	57.2±6.3	0.639
Spine BMD (g/cm^2)	0.86±0.13	0.99±0.15	<0.001
Spine BMC (g)	50.0±10.5	56.6±11.3	0.002
Spine T-score	-1.7±1.2	-0.5±1.3	<0.001
Femoral neck area	5.0±0.4	5.0±0.5	0.648
Femoral neck BMC (g)	3.4±0.5	3.7±0.5	0.002
Femoral neck BMD (g/cm^2)	0.67±0.08	0.73±0.96	0.001
Hip area (cm²)	34.6±3.4	35.2±3.4	0.312
Hip BMC (g)	27.7±4.6	31.2±5.0	<0.001
Hip BMD (g/cm^2)	0.80±0.10	0.88±0.11	<0.001
Hip T-score	-1.1±0.9	-0.5±0.9	<0.001
Whole body area (cm²)	1885.0±144.7	1991.9±127.5	<0.001
Whole body BMC (g)	2081.1±288.5	2280.4±338.0	0.001
Whole body BMD (g/cm^2)	1.10±0.10	1 .14±0.11	0.052
Whole body Total Fat Mass (kg)	21.5±4.9	33.9±6.2	<0.001
Whole body Total Lean Mass (kg)	38.3±4.0	42.0±4.3	<0.001
TFM/TLM Ratio	0.6±0.1	0.8±0.1	<0.001
Waist/Hip Ratio	0.8±0.1	0.8±0.1	<0.001
Stiffness index	87.0±12.7	90.0±14.0	0.235
QUS T-score	-0.8±0.8	-0.6±0.9	0.239
QUS Z-score	0.6±0.8	0.9±0.9	0.124
AEE (Cal/day)	5187.8±16967.4	1814.9±3858.7	0.092

SD=standard deviation; BMI=body mass index; WC=waist circumference; HC=hip circumference; QUS=Quantitative Ultrasound Sonometry; BMD=bone mineral density; BMC=bone mineral content; AEE=activity energy expenditure.

In Table 4-4, there were positive correlations between the body composition variables and all the BMD measures at different sites as well as the QUS T-score. Negative correlations were observed with age and all the BMD measurements and QUS T-score. On the other hand, LM had higher significant positive correlations with BMD at all sites except FM. In addition, high significant positive correlations were observed for weight and all BMD sites.

Table 4-4 Pearson correlation coefficients of body composition parameters and BMD

Parameters	Femoral neck BMD (g/cm^2)	Hip BMD (g/cm^2)	Spine BMD (g/cm^2)	Whole body BMD (g/cm^2)	QUS T-score
Age (years)	-0.282**	-0.271***	-0.023^{ns}	-0.151*	-0.262**
Weight (kg)	0.468***	0.537***	0.455***	0.305***	0.278**
Height (cm)	0.259**	0.196*	0.089^{ns}	0.219**	0.240**
WC	0.276**	0.387***	0.405***	0.141^{ns}	0.098^{ns}
HC	0.286**	0.349***	0.299***	0.145*	0.136^{ns}
Waist-Hip Ratio	0.157*	0.254**	0.312***	0.071^{ns}	0.024^{ns}
Body mass index (kg/m^2)	0.367***	0.463***	0.427***	0.205*	0.193*
Fat mass (g)	0.346***	0.446***	0.377***	0.191*	0.134^{ns}
Lean mass (g)	0.497***	0.495***	0.449***	0.406***	0.387***
Stiffness Index	0.551***	0.520 ***	0.410***	0.437***	0.999***
QUS T-score	0.549***	0.516***	0.408***	0.433***	1.000
QUS Z-score	0.479***	0.451 ***	0.411***	0.408***	0.953***
AEE (Cal/day)	0.103^{ns}	0.053^{ns}	0.185*	0.092^{ns}	0.247**

* $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$(one tailed). BMI=body mass index; WC=waist circumference; HC=hip circumference; QUS=Quantitative Ultrasound Sonometry; BMD=bone mineral density; AEE=activity energy expenditure; ns=not significant.

Lastly, to test if LM, FM, age, BMI and AEE significantly predicted BMD at all the sites in Table 4-5, multiple regression was used. The analysis shows that LM accounts for 24.7% of the variation in FN BMD [$F(1, 123) = 40.3$, $p < 0.001$]. Furthermore, the introduction of age explains an additional 5.4% of the variation [$F(2, 122) = 26.3$, $p < 0.001$]. For hip BMD, observations in Table 4-5 show that three predictors explained 35.5% of the variance [$F(3, 121) = 22.2$, $p < 0.001$]. It was found that LM significantly predicted hip BMD ($\beta = 0.348$, $p < 0.001$) as did FM ($\beta = 0.275$, $p<0.01$) and age ($\beta = -0.219$, $p<0.01$). Similarly, three predictors explained 28.5% of the variation for the spine BMD [$F(3, 121) = 16.0$, $p < 0.001$]. LM significantly predicted spine BMD ($\beta = 0.243$, $p < 0.05$) as well as BMI ($\beta = 0.325$, $p<0.01$) and AEE ($\beta = 0.196$, $p < 0.05$). Conversely, only LM explained 16.5% of the variability in whole body BMD [$F(1, 123) = 24.3$, $p < 0.001$]. The LM significantly predicted whole body BMD ($\beta = 0.406$, $p < 0.001$).

Table 4-5 Multiple regression analysis showing predictors of BMD

	B	SE B	95% CI B	β	R^2	*P*
			Femoral neck BMD			
Model 1					0.247	<0.001
Intercept	0.288	0.067	0.155, 0.421			
Lean mass	1.04×10^{-5}	0.000	0.000, 0.000	0.497		
Model 2					0.301	<0.001
Intercept	0.620	0.126	0.371, 0.869			
Lean mass	9.90×10^{-6}	0.000	0.000, 0.000	0.473		
Age	-0.005	0.002	-0.008, -0.002	-0.234		
			Hip BMD			
Model 1					0.245	<0.001
Intercept	0.350	0.080	0.192, 0.509			
Lean mass	1.24×10^{-5}	0.000	0.000, 0.000	0.495		
Model 2					0.307	<0.001
Intercept	0.367	0.077	0.214, 0.520			
Lean mass	9.22×10^{-6}	0.000	0.000, 0.000	0.369		
Fat mass	3.80×10^{-6}	0.000	0.000, 0.000	0.279		
Model 3					0.355	<0.001
Intercept	0.738	0.145	0.451, 1.025			
Lean mass	8.71×10^{-6}	0.000	0.000, 0.000	0.348		
Fat mass	3.75×10^{-6}	0.000	0.000, 0.000	0.275		
Age	-0.006	0.002	-0.009, -0.002	-0.219		
			Spine BMD			
Model 1					0.202	<0.001
Intercept	0.332	0.110	0.115, 0.550			
Lean mass	1.50×10^{-5}	0.000	0.000, 0.000	0.449		
Model 2					0.250	<0.001
Intercept	0.274	0.109	0.058, 0.490			
Lean mass	1.03×10^{-5}	0.000	0.000, 0.000	0.309		
BMI	0.009	0.003	0.003, 0.016	0.261		
Model 3					0.285	<0.001
Lean mass	8.12×10^{-6}	0.000	0.000, 0.000	0.243		
BMI	0.012	0.003	0.005, 0.019	0.325		
AEE	2.75×10^{-6}	0.000	0.000, 0.000	0.196		
			Whole body BMD			
Model					0.165	<0.001
Intercept	0.728	0.081	0.567, 0.889			
Lean mass	9.80×10^{-6}	0.000	0.000, 0.000	0.406		

BMI=body mass index; BMD=bone mineral density; AEE=activity energy expenditure; SE=standard error of the coefficient; CI=confidence interval.

4.5 Discussion

The results from this study indicate that there is a strong positive correlation between weight, BMI and regional (femoral neck, hip and spine) BMD, whole body BMD as well as the QUS T-score. Similarly, AEE was positively correlated with QUS T-score but not with the regional and whole body BMDs. LM not FM was found to be the strongest predictor of BMD at the regional sites and whole body. The multiple regression analysis showed that LM had positive regression weights, indicating that individuals with higher LM will be expected to have higher BMDs at all regional sites and whole body even after controlling for other variables in the model.

The BMI of the participants in this study is comparable to that of 'A Focus on Nutrition: Key Findings of the 2008/09 New Zealand Adult Nutrition Survey' [21]. According to WHO, 29.6% of adult females in Western Pacific are overweight compared to 24.1% in South-East Asia and 60.9% in the Americas [22]. In comparison with corresponding statistics, 29% of women in India, 27% in Oceania and 12.1-17.6% in Latin America are osteoporotic [23]. Although the populations need to be considered, the trend shows people with lower BMI are more likely to have bone health issues.

In this study, obesity was positively associated with bone mass. Meanwhile, age was identified as a co-predictor for FN and hip BMD, likewise BMI and AEE for spine BMD. Similarly, Salamat et al. [24] found a positive correlation between BMD and BMI indicators, giving additional evidence for the obesity paradox. Some studies have reported that obesity is positively associated with high bone mass [25, 26] probably as a result of the increased levels of hormones such as leptin, insulin, and oestrogen that are known to induce bone growth and inhibit the bone remodelling process. Other studies however have reported that obesity was negatively associated with bone mass [5, 27], possibly due to the differences in patterns and occurrence of obesity, fat distribution, and osteoporosis in men and women, and between pre- and postmenopausal women [6].

Furthermore, the results of this study show that LM alone accounts for 24.7% of femoral neck, 24.5% of hip and 20.2% of the spine BMDs' variability. These findings are similar to that of Casale et. al [13] and Sotunde et. al [12] in Pacific Island and black South African women respectively, indicating that LM is the strongest predictor of BMD. Our results also suggests that LM is more positively correlated with bone mass than the adipose tissue. A study [28] in Ukraine presented a similar result showing a positive correlation between the total LM and FN and spine BMD for women in the middle and late postmenopausal period. In addition, a study of postmenopausal women by Gnudi et. al [7] shows total LM and total FM were associated with BMD, BMC and height independent-BMD in Gucasian postmenopausal women.

Similar results were also observed in a study by Wang and colleagues [29], they found LM had a greater effect on BMD than FM in young women.

However, results of the present study are contrary to previous studies by Reid et. al [30-32], suggesting that the relationship between LM and BMD are artefacts. The differences in these results however could be explained by the meta-analysis of Khosla et al. [8] which found that both lean body mass and fat body mass have important effects on bone mass, depending on the bone mass parameter used, the skeletal site measured, and menopausal status.

Limitations of this study include its cross-sectional design and setting, thus preventing causal relationships and generalisation. The method of assessing physical activity was NZPAQ however, bone-specific physical activity questionnaire (BPAQ) has a current and past bone-related exercises. Furthermore, there was lack of other contributors and predictors of bone status such as diet, nutrients and vitamin D. These parameters are important for lean mass accumulation in postmenopausal women.

4.6 Conclusion

In conclusion, our findings suggest that LM was the strongest predictor of BMD at all sites amongst postmenopausal women. It is important that when considering prevention and/or management of osteoporosis, LM should be the target for improvement rather than FM reduction. In addition, it emphasises the significance of the accumulation of LM rather than FM in this age group. These findings will bring about further novel clinical research on the mechanisms by which LM regulates bone mass.

4.7 References

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GRADUATE RESEARCH SCHOOL

STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Bolaji Lilian Ilesanmi-Oyelere
Name/title of Primary Supervisor:	Professor Marlena C. Kruger
Name of Research Output and full reference:	
Ilesanmi-Oyelere, B.L.; Brough, L.; Coad, J.; Roy, N.; Kruger, M.C. The Relationship between Nutrient Patterns and Bone Mineral Density in Postmenopausal Women. Nutrients 2019	
In which Chapter is the Manuscript /Published work:	Chapter 5
Please indicate:	
• The percentage of the manuscript/Published Work that was contributed by the candidate:	85%
and	
• Describe the contribution that the candidate has made to the Manuscript/Published Work:	
Involved in the study design, participant recruitment, running of the study and data collection, analysis and interpretation and writing of the first manuscript draft	
For manuscripts intended for publication please indicate target journal:	
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Chapter 5 The Relationship between Nutrient Patterns and Bone Mineral Density in Postmenopausal Women

The objective of this part of the study was to determine the associations between nutrient patterns and nutrients obtained from foods entered into 3-Day Diet Diary (3DDD) and all BMD sites. This chapter explores the relationship between important nutrients and nutrient patterns that may influence bone health status in postmenopausal women.

This chapter is published as:

Ilesanmi-Oyelere, B.L.; Brough, L.; Coad, J.; Roy, N.C.; Kruger, M.C. The Relationship between Nutrient Patterns and Bone Mineral Density in Postmenopausal Women. *Nutrients* 2019, 11, 1262.

5.1 Abstract

In women, the menopausal transition is characterised by acid-base imbalance, estrogen deficiency and rapid bone loss. Research into nutritional factors that influence bone health is therefore necessary. In this study, the relationship between nutrient patterns and nutrients important for bone health with bone mineral density (BMD) was explored. In this cross-sectional analysis, 101 women aged between 54 and 81 years were eligible. Body composition and BMD analyses were performed using dual-energy X-ray absorptiometry (DXA). Nutrient data were extracted from a 3-day diet diary (3-DDD) using Foodworks 9 and metabolic equivalent (MET-minutes) was calculated from a self-reported New Zealand physical activity questionnaire (NZPAQ). Significant positive correlations were found between intakes of calcium ($p = 0.003$, r = 0.294), protein ($p = 0.013$, r = 0.246), riboflavin ($p = 0.020$, r = 0.232), niacin equivalent ($p = 0.010$, r = 0.256) and spine BMD. A nutrient pattern high in riboflavin, phosphorus and calcium was significantly positively correlated with spine ($p < 0.05$, r = 0.197) and femoral neck BMD ($p < 0.05$, r = 0.213), while the nutrient pattern high in vitamin E, α-tocopherol, β-carotene and omega 6 fatty acids was negatively correlated with hip ($p < 0.05$, r = −0.215) and trochanter BMD ($p < 0.05$, r = −0.251). These findings support the hypothesis that a nutrient pattern high in the intake of vitamin E, α-tocopherol and omega 6 fatty acids appears to be detrimental for bone health in postmenopausal women.

5.2 Introduction

Diet and hence nutrition are shown to be useful and modifiable tools for the management and possibly the prevention of metabolic bone disorders such as osteoporosis. This is especially true if good nutrition and a healthy balanced diet is introduced early in life, and followed through to adulthood [1,2]. In the past two decades, a growing body of literature has recognised the importance of nutrients in bone health [3,4] and dietary patterns [5]. However, few studies have investigated the impact of nutrient patterns on bone density [1,2]. Although investigating a single nutrient can be beneficial, research into a combination of nutrients in foods may help individuals with differing environment, culture, food habits and preferences putting into consideration the synergistic, additive and antagonistic effects of these nutrients when consumed together.

Many nutrients have been associated with increased BMD in many different populations. Nutrients readily available in fruits and vegetables (for example potassium and magnesium) have been linked with higher BMD in midlife women aged 45 – 55 years [6]. Studies have also linked an increase in calcium, phosphorus, magnesium and vitamin D intakes with greater BMD in postmenopausal women [7,8] as well as indicating an association between high protein intake and reduced incidence of fracture [9].

Results of the relationship between levels of homocysteine and B-vitamins, especially riboflavin and niacin, are few and inconsistent [10,11].

The Western diet, typically lacking alkali-forming capability provided by high fruit and vegetable intakes, has been considered a factor for the higher prevalence of osteoporosis in the developed world [12]. This diet, also high in saturated fatty acids, may affect the bioavailability and bio-accessibility of calcium forming poorly digestible calcium-fatty acid soaps. Studies have also reported monounsaturated fatty acids (MUFAs) and polyunsaturated fatty acids (PUFAs) as negatively associated with BMD [12–14]. These findings were concluded to be a result of either hyperinsulinemia induced by a high fat diet leading to a negative calcium–magnesium balance or a reduced calcium absorption as a result of a high lipid diet resulting from the formation of calcium soaps [12].

The effects of the acid-base imbalance due to an increased consumption of a high fat diet might not be as pronounced in women who are still menstruating [12]. However, in postmenopausal women, an increase in steady state acid level with age [15] and oestrogen deficiency, needs to be balanced with an increased intake of fruits and vegetables [6,12]. In postmenopausal women, it has been suggested that an increased acidity with age coupled with a high fat nutrient pattern may therefore be detrimental to BMD [12,16]. Diet high in phosphorus and proteins and sulphur amino acids may also induce low-grade metabolic acidosis, thereby increasing dietary acidic load [17].

The influence of nutrient patterns on postmenopausal bone loss is not well understood as most research has focused on calcium, vitamin D and single nutrients but there is little work on nutrient patterns. In addition, older studies have used mainly food frequency questionnaires (FFQ) rather than diet records (which are considered to be the gold standard method) and many did not adjust for confounders such as body mass index (BMI) and physical activity. The aim of this current research was to investigate the relationship between nutrient patterns and also individual nutrients important for bone health with BMD in post-menopausal women.

5.3 Materials and Methods

5.3.1 Study Design

A total of 127 postmenopausal women aged between 54 and 81 years were recruited to participate in the cross-sectional study, "Bugs'n'Bones", that took place in the Human Nutrition Research Unit of Massey University, Palmerston North campus from June to December, 2017. The sample size was calculated using G*Power software version 3.0.10 and eighty-eight participants were required for a 90% power and an alpha of 5% for a T-test, to test for the mean differences between two groups of osteoporotic and non-osteoporotic women based on the BMD values. Of the 127 women, a total of 110 women's data were evaluated.

Participants were recruited by advertisement on campus, the Whanganui Chronicle and by using a recruitment agency, Trial Facts (https://trialfacts.com/). Women at least 5 years post-menopause based on menstruation were included in the study. Exclusion criteria were presence of any systemic disease, food intolerances that affect the gastrointestinal tract, smokers and high intake of alcohol (more than 4 standard drinks per week). Participants with significant weight loss or weight gain (5kg) within the past year were excluded. Two participants were excluded post recruitment, one due to following a ketogenic diet and the other due to health conditions, while nine participants had missing diet records.

Evaluation of energy intake: basal metabolic rate using the Goldberg cut-off [18] revealed 3 (3%) over-reporters, 12 (10%) under-reporters and 101 (87%) plausible reporters. A total of 26 participants were therefore excluded from the study. All participants were free living and healthy. Written informed consent was obtained from participants before commencing data collection. The study was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) by the number ACTRN12617000802303. This study was also approved by Massey University Human Ethics Committee: Southern A, Application 17/17.

5.3.2 Anthropometric and Body Composition Measurements of the Participants

Fasting participants' body weight was measured using the Detecto 437 eye-level weigh beam physician scale (USA) to the nearest 0.1 kg and standing height was measured using a wall-mounted stadiometer to the nearest 0.1 cm wearing light clothes and no shoes; BMI was calculated as weight divided by height squared (kg/m^2). The waist to hip ratio was determined by measuring the waist and hip circumference to the nearest 0.1 cm using a non-stretchable measuring tape. The waist to hip ratio was calculated as a marker of abdominal obesity. Body composition measurements, FM, LM and fat percentage were measured and analysed using the Hologic QDR series Discovery A, bone densitometry system [Dual energy X-ray Absorptiometry (DXA)]. BMD was measured at the femoral neck (FN), lumbar spine (LS) [L1-L4], total hip and whole body.

All measurements were conducted on the non-dominant parts of the body. The DXA machine was calibrated every morning for all the measurements and at the end of each day. The *in vivo* reproducibility of the coefficient of variation ranged between 0.34 and 0.70% for all measured sites. The reported BMD values were calculated as means of four measured values from L1–L4. Apex System Software version 4.5.3 (USA) was used for analysing the DXA scans. Osteoporosis was defined as a T score ≤ 2·5 and osteopenia as T score between −1.0 and −2.5 according to the WHO criteria [18].

5.3.3 Dietary Intake Assessment

Participants' dietary assessment of the food patterns was investigated with a 3-day diet diary (3-DDD) over non-consecutive days (including one weekend day). The 3-DDD was used to collect information on participants' food and beverage intake utilising household measures. The principal investigator showed the participants examples of the household measures (e.g. measuring cups and spoons) available in the Human Nutrition laboratory at Massey University.

The 3-DDD has been recommended to get information on mean food consumption with an advantage as the 'golden standard' for dietary assessment [19]. Brand name of food products, recipes and method of food preparation were recorded. Food works 9 professional, Xyris software was used to analyse the participants' diet data.

5.3.4 Physical Activity Questionnaire

Levels of physical activity were assessed using the New Zealand Physical Activity Questionnaire - short form (NZPAQ-SF) [20]. The NZPAQ has previously been validated by Boon et al. [21]. Physical activities were then quantified by METs -min/day which was calculated by using the scoring protocol of IPAQ for continuous score. MET values and formula for computation of MET-minutes was calculated and used as below [22].

Walking MET-minutes/week at work = 3.3 × walking minutes × walking days at work.

Moderate MET-minutes/week at work = 4.0 × moderate-intensity activity minutes × moderate intensity days at work.

Vigorous MET-minutes/week at work = 8.0 × vigorous-intensity activity minutes × vigorous intensity days at work Total Work MET-minutes/week = sum of Walking + Moderate + Vigorous MET-minutes/week scores at work.

Total PA MET-minutes/week = sum of walking + moderate + vigorous MET-minutes/week scores.

5.3.5 Nutrient Pattern Identification

Principal component analysis (PCA) was used to identify nutrient patterns using 36 nutrients collated from all measured nutrients. The analysis was performed for 101 study participants in order to reflect the nutrient patterns. Orthogonal (varimax) rotation was performed to reduce the correlations between factors and increase interpretability [2].

5.3.6. Data Analyses

IBM SPSS version 25 (IBM Company, Armonk, NY, USA) was used for all statistical analyses. The outcome variables used were BMD of the whole body and at skeletal sites. The values of all variables for the whole body and regional sites were presented as mean ± standard deviation. The independent variables consist of the nutrient intakes such dietary calcium, phosphorus, fat, protein and the nutrient patterns obtained from the dimension reduction of 36 nutrients. Variables were parametric and normally distributed. Correlation analyses of the whole body, regional sites BMD with the independent variables were performed to obtain the Pearson's correlations. All *p*-values were reported significant at 0.05 or less.

5.4 Results

The mean age of the participants was 63 years (range = 54, 81years) and the mean (SD) spine BMD was 0.94 (0.15) g/cm^2 (Table 5-1).

Table 5-1 General characteristics and nutrient intakes of 101 participants based on 3-DDD.

Parameters	Mean±SD (*n* = 101)	Range	
		Min	Max
Age (years)	62.9 ± 4.4	54	81
BMI (kg/m^2)	26.3 ± 4.2	17.9	44.0
Waist-Hip ratio	0.8 ± 0.1	0.7	1.1
Spine (L1-L4) BMD (g/cm^2)	0.9 ± 0.2	0.5	1.3
Spine (L1-L4) T-score	−1.0 ± 1.4	−4.6	2.6
Femoral neck BMD (g/cm^2)	0.7 ± 0.1	0.5	1.0
Total hip BMD (g/cm^2)	0.9 ± 0.1	0.6	1.2
Total hip T-score	−0.7 ± 1.0	−2.5	2.1
WB BMD (g/cm^2)	1.1 ± 0.1	0.9	1.5
Energy Intake (kJ)	8109.8 ± 1727.5	4939.7	13843.4
Protein (g)	85.6 ± 21.5	42.4	155.7
Total fat (g)	79.5 ± 22.4	33.2	128.9
Carbohydrate (g)	192.0 ± 64.4	61.8	394.6
Saturated fat (g)	30.5 ± 11.8	8.3	66.4
Polyunsaturated fat (g)	12.1 ± 6.0	3.4	48.8
Monounsaturated fat (g)	27.2 ± 8.7	11.0	51.3
Cholesterol (mg)	295.0 ± 146.1	18.2	753.5
Sugars (g)	96.1 ± 38.5	29.6	183.3
Starch (g)	95.8 ± 37.4	24.4	215.6
Dietary fibre (g)	27.2 ± 9.0	9.9	53.0
Vitamin C (mg)	118.2 ± 69.4	13.6	387.1
Vitamin D (µg)	6.3 ± 6.5	0.3	33.2
Vitamin E (mg)	10.6 ± 4.0	2.6	20.3
Vitamin B6 (mg)	2.4 ± 1.1	0.8	8.1
Vitamin B12 (µg)	4.2 ± 3.8	1.1	31.0
Vitamin K (µg)	19.9 ± 19.0	0.0	90.7
Vitamin A (µg)	1038.4 ± 860.5	291.1	8085.9
Riboflavin (mg)	2.1 ± 0.8	0.9	4.7
Alpha tocopherol (mg)	8.3 ± 3.2	2.4	17.2
Niacin equivalent (mg)	37.7 ± 10.5	16.7	66.6
Calcium (mg)	929.3 ± 358.9	336.7	2170.3
Sodium (mg)	2058.4 ± 816.7	616.2	5073.2
Phosphorus (mg)	1520.4 ± 383.1	810.2	2639.3
Iron (mg)	12.4 ± 5.3	4.7	48.2
Zinc (mg)	10.3 ± 3.2	4.9	20.6
Magnesium (mg)	372.2 ± 97.6	211.6	592.1
Potassium (mg)	3613.6 ± 933.2	1930.0	6448.8
Phytosterols (mg)	21.0 ± 42.6	0.0	218.2
Caffeine (mg)	251.0 ± 218.9	0.0	1834.1

BMI=body mass index; WB=whole body; BMD=bone mineral density.

Three nutrient patterns were generated by PCA accounting for a total of 45.4% explained variance of the nutrient intakes (Table 5-2).

Table 5-2 Factor loading matrix for the nutrient patterns of 101 participants.

	Nutrient Pattern * Factor Loadings		
	NP1	**NP2**	**NP3**
Riboflavin_mg	**0.829**		
Phosphorus_mg	**0.798**	0.106	**0.346**
Calcium_mg	**0.777**		
Sugars_g	**0.774**		
Potassium_mg	**0.765**	0.218	
Vitamin B6_mg	**0.759**		
Carbohydrate_g	**0.755**		0.110
Magnesium_mg	**0.700**	**0.450**	0.229
Thiamin_mg	**0.538**		−0.241
Sodium_mg	**0.529**		0.165
Iron_mg	**0.524**	0.259	0.164
Iodine_µg	**0.417**	0.130	
Niacin equivalent_mg	**0.392**		0.140
Vitamin B12_µg	**0.346**	0.101	
Retinol_µg			
Polyunsaturated fat_g		**0.811**	0.264
Vitamin E_mg	0.103	**0.807**	0.163
Alpha tocopherol_mg	0.114	**0.779**	0.116
Linoleic acid_g		**0.686**	0.178
Beta carotene_µg	0.291	**0.576**	**−0.410**
Alpha linolenic_ALA_g		**0.535**	0.138
Alpha_carotene_µg	**0.361**	**0.516**	**−0.350**
Eicosapentaenoic_EPA_g		**0.483**	
Docosahexaenoic_DHA_g	−0.168	**0.470**	
Vitamin A_µg	0.185	0.255	−0.171
Total fat_g	0.194	**0.373**	**0.776**
Monounsaturated fat_g		**0.499**	**0.708**
Oleic acid_g	0.114	**0.449**	**0.707**
Saturated fat_g	0.285	-0.113	**0.681**
Protein_g	**0.520**		**0.588**
Zinc_mg	**0.432**	0.125	**0.558**
Cholesterol_mg			**0.548**
Biotin_µg		0.121	**0.524**
Pantothenic acid_mg	0.227		**0.374**
Vitamin C_mg	0.279	0.202	**−0.339**
Erucic acid_g			0.133

NP, nutrient pattern; * Nutrient patterns were extracted by principal component analysis. Only factors loading >|0.1| are displayed. The bold text indicates a factor loading >|0.3|.

Nutrient pattern NP1 was characterised by high levels of riboflavin, phosphorus, calcium and explained the variance of 24.1% while NP2 was characterised by high levels of dietary fats and fatty acids explaining the variance of 12.4% of the nutrient intake. In addition, NP3 characterised by high levels of vitamin A, fat and low levels of vitamin C and β−carotene explained the nutrient intake variance of 9.0%. Nutrient intakes across quartiles of each nutrient pattern are as shown in Appendix 1 and 2.

There was a trend of positive correlation between NP1 and all of the BMD sites even after adjusting with age, BMI and MET-minutes (Table 5-3). However, NP2 was mainly negatively correlated with the BMD sites. Nutrient pattern NP3 was also positively correlated with the BMD sites without adjustments but became negatively correlated when adjusted for BMI and MET-minutes. The confounding variables were adjusted for age because of the age range while BMI and MET-minutes was adjusted for due to the impact on BMD. The NP1 high in riboflavin, calcium and phosphorus was positively correlated with the spine BMD ($p = 0.048$, r = 0.197) and femoral neck BMD ($p = 0.033$, r = 0.213), while NP2 high in fats, vitamin E and fatty acids was significantly negatively correlated with total hip ($p = 0.031$, r = −0.215).

Table 5-3 Correlations of nutrient pattern scores and bone mineral density for 101 participants.

		Bivariate Correlations		
Nutrient Pattern	**Spine BMD**	**FN BMD**	**Hip BMD**	**WB BMD**
NP1	0.197 *	0.213 *	0.103	0.194 *
NP2	−0.030	−0.175	−0.215 *	−0.115
NP3	0.112	0.103	0.172	0.168
		Partial correlations adjusting for age		
NP1	0.198 *	0.186	0.073	0.177
NP2	−0.030	−0.193	−0.233*	−0.124
NP3	0.112	0.107	0.178	0.171
		Partial correlations adjusting for age and BMI		
NP1	0.261 **	0.240 *	0.133	0.199 *
NP2	0.062	−0.127	−0.158	−0.089
NP3	−0.061	−0.048	−0.009	0.107
		Partial correlations adjusting for age, BMI and MET		
NP1	0.211 *	0.213 *	0.111	0.184
NP2	0.100	−0.111	−0.147	−0.079
NP3	−0.059	−0.046	−0.007	0.109

FN=femoral neck; WB=whole-body; *Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed).

There was a trend of positive correlations across the nutrients high in the NP1 as well as the calcium/phosphorus ratio and niacin equivalent (Table 5-4). Interestingly, niacin equivalent and riboflavin had a strong positive effect on the BMD sites for the post-menopausal women even after adjustment for confounders. Calcium (p = 0.003, r = 0.294) and niacin equivalents (p = 0.010, r = 0.256) were weakly positively correlated with the spine BMD. These positive correlations were mainly significant for the spine and whole-body BMD.

Table 5-4 Correlations between selected nutrient intakes, calcium/phosphorus ratio and bone mineral density.

	Bivariate Correlations			
	Spine BMD	**FN BMD**	**Hip BMD**	**WB BMD**
Calcium intake	0.294 **	0.206*	0.148	0.239 *
Phosphorus intake	0.189	0.147	0.076	0.219 *
Ca/P ratio	0.257 *	0.160	0.154	0.165
Protein intake	0.246 *	0.182	0.178	0.241 *
Riboflavin intake	0.232 *	0.194	0.143	0.193
Niacin eq. intake	0.256 **	0.305 **	0.257 **	0.299 **
Partial correlations adjusting for age				
Calcium intake	0.295 **	0.187	0.127	0.226 *
Phosphorus intake	0.189	0.125	0.052	0.206 *
Ca/P ratio	0.257 *	0.146	0.140	0.155
Protein	0.246 *	0.181	0.177	0.239 *
Riboflavin intake	0.233 *	0.167	0.113	0.175
Niacin eq. intake	0.256 *	0.294 **	0.244*	0.290 **
Partial correlations adjusting for age and BMI				
Calcium intake	0.309 **	0.188	0.127	0.224 *
Phosphorus intake	0.184	0.114	0.030	0.200 *
Ca/P ratio	0.282 **	0.157	0.158	0.158
Protein	0.153	0.089	0.059	0.199 *
Riboflavin intake	0.300 **	0.219 *	0.179	0.198 *
Niacin eq. intake	0.211 *	0.256 *	0.194	0.268 **
Partial correlations adjusting for age, BMI and MET-minutes				
Calcium intake	0.251 *	0.152	0.099	0.207*
Phosphorus intake	0.117	0.076	0.000	0.183
Ca/P ratio	0.259 *	0.140	0.145	0.148
Protein	0.131	0.075	0.048	0.192
Riboflavin intake	0.244 *	0.187	0.155	0.180
Niacin eq. intake	0.212 *	0.254 *	0.192	0.267 **

FN=femoral neck; Troch=trochanter; WB=whole body; * Correlation is significant at the 0.05 level (2-tailed); ** Correlation is significant at the 0.01 level (2-tailed).

5.5 Discussion

This study indicates that a nutrient pattern higher in calcium, phosphorus, potassium, protein and the B vitamins (NP1) was positively correlated with whole body and skeletal sites BMD A nutrient pattern higher in fats, vitamin E and fatty acids (NP2) was negatively correlated with BMD at all sites. Univariate analysis also showed positive correlations between intakes of calcium, protein, riboflavin, niacin equivalent and BMD of the spine. Calcium, riboflavin and niacin intakes were still positively related with spine BMD even after adjusting for age, BMI and MET-minutes. The calcium–phosphorus ratio was positively associated with spine BMD.

Calcium, phosphorus, potassium and protein are well-known to have a beneficial effect on bone health [4,6,12,14], however, little research is available on the role of niacin and riboflavin on bone health, especially in older women. Although these B vitamins may not have a direct role in bone metabolism, their role in energy metabolism and as an essential cofactor for the enzyme ornithine decarboxylase plays a part in osteoblast NADPH concentrations for the vitamin K cycle [23]. Riboflavin, also referred to as vitamin B2, is a water-soluble vitamin important for cell growth and function, while niacin is important for keeping the bone stronger by retention of calcium [23,24]. Milk and dairy products have been reported as the greatest contribution to riboflavin intake in the Western diet [24].

Low or inadequate levels of vitamin B12, folate and vitamin B6 in the metabolism of homocysteine (Hcy) are known to cause hyper-homocysteinemia which is considered to be detrimental to bone health [25,26]. The mechanism linking increased homocysteine to increased risk of fracture has not yet been clarified, however, elevated levels of homocysteine have been shown to affect the cross-linking of collagen, thereby reducing bone strength [27,28].

The results of this study are similar to those of Shono et al. 1997 which found the effectiveness of riboflavin in maintaining a higher bone density in premenopausal Japanese women [29]. Furthermore, a similar study identified a significant positive correlation between niacin and BMD [30]. The NP1 was also high in vitamin B12 which is known as an important cofactor for osteoblast-related proteins such as osteocalcin and alkaline phosphatase [23]. It has therefore been suggested that it is important to set an appropriate recommended levels of B vitamins for optimal bone health [24].

The nutrient pattern (NP2) was high in fats, vitamin E and alpha-tocopherol and was mainly negatively correlated with BMD. Intake of fat and its influence on calcium absorption has also been reported with MUFAs and PUFAs, indicated as negatively associated with BMD [12–14]. Evidence as far back as the early 1900s suggests that fat interferes with calcium absorption through the formation of calcium soaps [31] which might be a reason for the negative relationship between fats and BMD. It has also been suggested that the possible mechanism for the negative associations between fat consumption and BMD may be as a result of high-fat-induced hyper-insulinema that may lead to a negative calcium–magnesium balance [12].

In addition, studies have reported the role of serum vitamin E and alpha tocopherol in bone mass maintenance through the induction of osteoclast fusion in a mouse model *in vivo* and *in vitro* [32] and in a rat model [33]. This is, however, in contrast to the findings of Carvalho et al. 2013 [34], indicating that vitamin E does not prevent alveolar bone loss in another animal model. A large longitudinal study of 891 premenopausal women, conducted by Macdonald et al. 2004 [12], resulted in mixed outcomes; total vitamin E intake of dietary plus supplementation correlated positively with BMD, but was not significant while dietary vitamin E intake was significantly negatively correlated with BMD. The role and effect of vitamin E on bone is therefore not conclusive, as is supported by a review by Mohamed et al. [35].

Meanwhile, the nutrient pattern characterised by a high fat, protein, cholesterol and low in vitamin C (NP3) was positively correlated with BMD when performed in the crude state and when adjusted for age but negatively correlated when adjusted for BMI and Met-minutes. Accounting for the effect of age, BMI and MET-minutes, the NP3 was negatively associated with BMD except the whole body BMD and these were not significant. This is consistent with the findings by Melaku et al. 2017 [2] who found that an animal-sourced nutrient pattern high in fats, protein and cholesterol was positively associated with BMD independently but negatively associated when adjusted for BMI and energy intake and in likewise manner was not significant. This finding could be a result of the effect of energy intake and physical activity which can be observed as confounders in this study.

The strength of this study includes the use of a 3DDD in the analysis of the data as well as the use of Goldberg cut-offs for identifying plausible reporters for the exclusion of under- and over-reporters of diet. Limitations of the study are its cross-sectional nature and the small sample size which may not allow generalisation or cause and effect principle to be applied.

In summary, we found that a nutrient pattern high in fats, omega 6 fatty acids, alpha tocopherol and vitamin E was negatively correlated with BMD while a nutrient pattern high in calcium, phosphorus, riboflavin and niacin was positively correlated with BMD at all sites. The link between saturated fatty acids and a possible reduced calcium absorption due to calcium-fatty acid soaps formation needs more in-depth research. However, the limitations of this study are to be considered when interpreting the findings of this study. It should be noted that the high intakes mentioned are relative to the nutrient pattern in our cohort and intakes of protein must be regulated for the mild metabolic acidosis induced by overconsumption of sulfur-containing amino acids. Further research is therefore needed for longitudinal studies to support the nutrient pattern approaches, especially ones high in vitamin E, β-carotene and omega 6 fatty acids and its impact on BMD and osteoporosis for both clinical and public health interventions.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Bolaji Lilian Ilesanmi-Oyelere
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Name of Research Output and full reference:	
Ilesanmi-Oyelere, B.L.; Brough, L.; Coad, J.; Roy, N.; Kruger, M.C. Dietary patterns, body composition and bone health in New Zealand postmenopausal women	
In which Chapter is the Manuscript /Published work:	Chapter 6
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and	
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Chapter 6 Dietary patterns, body composition and bone health in New Zealand postmenopausal women

In chapter 5, calcium, protein and B-vitamins were found to be positively associated with BMD sites while a nutrient pattern with a high intake of vitamin E, alpha-tocopherol, beta-carotene and omega 6 fatty acids appeared to be detrimental. The objective of this investigation was to determine the associations between New Zealand postmenopausal women's dietary patterns and whole-body BMD and T-scores of hip, FN and spine.

This chapter is for submission to Frontiers in Nutrition

6.1 Abstract

Nutrition affects bone health status. However, dietary patterns generate insight into which particular combination of foods may influence nutritional status and bone health. The aim of this study was to explore the associations between dietary patterns, bone mineral density (BMD) and T-scores, and body composition in New Zealand postmenopausal women.

This cross-sectional study examined 125 postmenopausal women aged between 54 - 81 years. Body composition, BMD and T-scores were determined using dual-energy X-ray absorptiometry (DXA). Diet composition was assessed using a validated food frequency questionnaire (FFQ) composed of 108 food items, from which 34 food groups were created. Dietary patterns were identified by principal component analysis. The bone and body composition data including skeletal sites T-scores, waist circumference, BMI and body fat percentage were regressed onto the dietary patterns.

Four dietary patterns were identified; the milk and milk-based beverages dietary pattern, the dessert, cheese and red meat dietary pattern, the fruit-rich, biscuit and crackers dietary pattern and the oily fish, sports drink and seafood-rich dietary pattern. The milk and milk-rich beverages dietary pattern was significantly positively associated with spine T-score (r = 0.247, $P = 0.008$), and whole-body BMD (r = 0.182, $P = 0.051$). An oily fish, sports drink and seafood-rich dietary pattern was negatively associated with waist circumference (r = -0.157, $P = 0.094$), body mass index (r = -0.163, $P = 0.081$) and body fat percentage (r = -0.247, $P = 0.008$).

A dietary pattern characterised by a high factor loading of milk and milk-rich beverages was positively associated with whole-body BMD and spine T-score, while an oily fish, sports drink, seafood-rich dietary pattern was inversely associated with total body fat percentage. Consumption of milk, even with coffee had an impact on bone health among the women. Further intervention research is warranted to confirm relationships between dietary patterns and skeletal sites such as hip and femoral neck T-scores.

6.2 Introduction

Extensive research studies have reported the relationship between dietary factors and its effect on bone health [1-3]. Consumption of high amount of fruits and vegetables [1], milk, dairy products and green tea [4] as well as the Mediterranean diet [5] has been associated with lower risk of osteoporosis. Likewise plant-based diets have been linked to weight loss [6] while meat-based diets were linked to higher blood pressure [7].

Bone health in older age is a public health issue especially for populations at risk such as the Caucasians in comparison to their non-Caucasian counterparts. The risk is heightened for postmenopausal women with oestrogen deficiency related to both the early and late stages of osteoporosis [8]. A modifiable mechanism of reducing the effect of menopause on older women is the consumption of a nutrient dense dietary pattern. Dietary patterns rich in nutrients such as calcium, phosphorus, protein and vitamin D have been documented to have a beneficial role for bone mass [9]. However, in order to better understand the relationship between dietary patterns and bone health across population in different geographical locations, data from different populations of postmenopausal women are important.

Specific diets play a significant role in determining nutritional status and health outcomes, it is therefore imperative to study what patterns of diet affect or contribute to an individual's well-being. Although few studies have investigated the relationship between dietary patterns and body composition [10, 11], no study has emerged from New Zealand in relation to dietary patterns and BMD. In New Zealand, there are limited studies that have explored the relationships between dietary patterns and nutritional status [12, 13]. Therefore, to support the search for an 'ideal' diet, studies from various communities are warranted to enable generalisation. To our knowledge no study has previously analysed the relationship between dietary patterns and bone health in New Zealand postmenopausal women.

We consequently investigated the associations between dietary patterns and bone health as well as nutritional status in New Zealand postmenopausal women. This was assessed by determining the body composition using DXA and FFQ for the dietary data. The objectives were to determine the relationships between New Zealand-specific dietary patterns and BMD site T-scores as a measure of bone density along with the body composition of postmenopausal women.

6.3 Materials and Methods

6.3.1 Study design

A total of 127 postmenopausal women aged between 54 and 81 years were recruited to participate in the cross-sectional study; "Bugs'n'Bones" that took place in the Human Nutrition Research Unit of Massey University, Palmerston North campus from June to December 2017. Two participants were excluded post recruitment, one due to consuming a ketogenic diet and the other due to health conditions. The sample size was calculated using G*Power software version 3.0.10 with a 90% power and an alpha of 5%.

Participants were recruited by advertisement on campus, the Whanganui Chronicle and by using a recruitment agency; Trial Facts (https://trialfacts.com/). The inclusion criteria were menopause of at least 5 years based on no menstruation. Exclusion criteria were presence of any systemic disease, food intolerances that affect the gut, smokers and high intake of alcohol. Participants with significant weight loss or weight gain within the past year were excluded. All participants were free living and apparently healthy.

Written informed consent was obtained from participants before commencing data collection. The study was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) with the number ACTRN12617000802303. This study was carried out in accordance with the recommendations of Massey University Human Ethics Committee Guidelines, Massey University Human Ethics Committee: Southern A, Application 17/17.

6.3.2 Anthropometric and body composition measurements of the participants

Participants' body weight was measured using the Detecto 437 eye-level weigh beam physician scale to the nearest 0.1kg. Standing height was measured using a stadiometer to the nearest 0.1cm wearing light clothes and no shoes on. Body mass Index (BMI) was calculated as weight divided by height squared (kg/m^2). Waist to hip ratio was determined by measuring the waist and hip circumference to the nearest 0.1cm using a non-stretchable measuring tape. Waist to hip ratio was calculated as a marker of abdominal obesity.

Body composition measurements, FM, LM and fat percentage were measured and analysed using the Hologic QDR series Discovery A, Bone densitometry system [Dual energy X-ray Absorptiometry (DXA)]. BMD was measured at the femoral neck (FN), lumbar spine (LS) [L1-L4], trochanter, Ward's triangle and total hip. The DXA machine was calibrated every morning for all the measurements and at the end of each day.

The reproducibility of the coefficient of variation ranged between 0.34 - 0.70% for all measured sites. The reported lumbar spine BMD values were calculated as means of four measured values from L1–L4. The Apex System Software version 4.5.3 was used for analysing the DXA scans. Osteoporosis was defined as a T score ≤ 2·5 and osteopenia as T score between -1.0 and -2.5 according to the WHO criteria [14].

6.3.3 Dietary intake assessment and dietary pattern identification

Participants' diets were assessed with a semi-quantitative FFQ. The FFQ was used to collect information on participants' frequency of food intake and beverage intake. Portion size, food and beverage intake were entered into the Foodworks version 9 Xyris software which was used to analyse the participants' diet data and nutrient analyses of intake were calculated.

Principal component analysis (PCA) is an acceptable exploratory factor analysis for linking a set of variables into smaller dimensions, while varimax rotation enables an orthogonal (uncorrelated) factors that are interpretable. Validated FFQs are a gold standard method of collecting information on quantity and frequency of foods consumed retrospectively [15]. Dietary assessment tools are therefore necessary for identifying patterns of an individual's diet in relation to any health issues associated.

PCA was used to identify dietary patterns using 34 food groups collated from a 108-item semi-quantitative FFQ. The analysis was performed for 125 study participants in order to reflect their dietary patterns. Varimax orthogonal rotation with Kaiser normalisation was performed to reduce correlations between factors and increase interpretability. Kaiser-Meyer-Olkin measure of sampling adequacy was 0.5 while Bartlett's test of sphericity was significant (<0.001).

6.3.4 Data analyses

IBM SPSS version 25 (IBM Company, Armonk, NY, USA) was used for all statistical analyses. The outcome variables used were BMD of whole body and the regional skeletal sites T-score and the body composition parameters. The values of all variables for the whole body and regional sites were presented as mean ± standard deviation. The independent variables consisted of the food intakes such as milks (all types of milk), fruits, vegetables, yoghurt/cream, white fish, red meat, coffee etc. The dietary patterns were obtained from the dimension reduction of 34 food items. Bone health and body composition parameters (the outcome variables) were regressed onto the dietary patterns generated. All p-values were reported significant at 0.05 or less.

6.4 Results

The results from Table 6-1 illustrate that the postmenopausal women had an average age of 62 years. The mean BMI was 27.9 kg/m² for the healthy and 24.9 kg/m² for the women with osteoporosis. The Table also shows the BMD and T-scores for the skeletal sites differentiating healthy from osteopenic/osteoporosis based on the spine T-score classification.

Table 6-1. Characteristics of participants

Parameters	**Healthy (60) Mean±SE**	**Osteopenic/osteoporotic (65) Mean±SE**	***P*-value**
Age (years)	62.32±0.64	62.91±0.49	0.463
BMI (kg/m²)	27.87±0.55	24.88±0.44	<0.001
Spine BMD	1.07±0.10	0.82±0.08	<0.001
Spine T-score	0.23±0.91	-2.02±0.09	<0.001
Hip BMD	0.92±0.01	0.79±0.01	<0.001
Hip T-score	-0.18±0.90	-1.21±0.09	<0.001
Femoral neck BMD	0.76±0.09	0.66±0.07	<0.001
Femoral neck T-score	-0.74±0.11	-1.69±0.08	<0.001

BMI=body mass index; BMD=bone mineral density; SE=standard error

An overview of the dietary pattern factor loadings of the 34 food groups identified from the FFQ is provided (Table 6-2). The dietary pattern (DP) 1 consist of a high factor loading of milk and milk-rich beverages and DP 2 is high in desserts, cheese and red meat. DP 3 comprises of high factor loadings of fruit, biscuits and crackers, potato and bread making it carbohydrate-rich while DP 4 is composed of high factor loadings of oily fish, sport drinks and seafood. These dietary patterns have been named based on the factor scores high loading as shown in Table 6-3.

Table 6-2 Factor loadings of dietary patterns

	Dietary Patterns			
Foods	1	2	3	4
Milks	**0.600**			
Vegetables	**-0.534**		0.286	-0.147
Offals	**-0.478**	0.188	-0.158	-0.110
Coffee	**0.462**	0.102	-0.148	-0.145
Malt and chocolate beverage	**0.450**		0.211	
Soup	**-0.430**	0-.287	0.414	
White fish	**-0.416**		0.277	0.123
Seafood	**-0.394**	0.367		**0.313**
Muesli and chocolate bars	**0.304**	0.257		-0.105
Pizzas/burgers	0.295	0.161		-0.152
Spirits	0.249	0.227		
White meat	-0.233			-0.172
Dessert	0.129	**0.718**	0.194	
Cheese	-0.174	**0.697**	0.107	
Red meat	-0.106	**0.491**		-0.286
Sauces/dressings	-0.261	**0.425**		-0.252
Carbonated drinks		**0.387**		
Juice	0.116	**0.360**		0.183
Wine		**0.304**	-0.216	-0.129
Fruit	0.302		**0.467**	
Biscuits and crackers	-0.102		**0.467**	-0.104
Spreads			**0.444**	0.179
Crisps/nuts	-0.116	-0.109	**0.440**	
Tin/dry fruit			**0.401**	
Tea	-0.312	0.120	**0.400**	
Yoghurts and cream	0.243	0.359	**0.373**	0.274
Rice/pasta		0.235	**0.343**	0.135
Bread			**0.338**	-0.200
Porridge	0.191		0.213	
Cake and Pie			-0.202	-0.188
Oily fish (Sardine and Tuna)		-0.181		**0.763**
Sport drinks	0.120	0.103		**0.742**
Potato	0.333	-0.128	0.320	**-0.413**
Beer				0.228

Extraction Method: Principal Component Analysis.

Rotation Method: Varimax with Kaiser Normalisation.[a]

Rotation converged in 9 iterations.

In Table 6-3, an outline of the names of the various DPs are shown according to their factor loadings below. Four DPs namely, milk and milk-rich beverages DP, dessert, cheese and red meat DP, fruit-rich, biscuit and crackers DP and oily fish, sports drink and seafood-rich DP were identified. A total of 28.1% of the variability in food intake was explained by the four dietary patterns. DP1 explained 8.2%, DP2 accounted for 7.8%, DP3 explained 6.3%, and DP4 accounted for 5.9% of the variability.

Table 6-3. Definitions of dietary patterns

Dietary Patterns	Names
Dietary pattern 1	Milk and milk-rich beverages dietary pattern
Dietary pattern 2	Dessert, cheese and red meat dietary pattern
Dietary pattern 3	Fruit-rich, biscuit and crackers dietary pattern
Dietary pattern 4	Oily fish, sports drink and seafood-rich dietary pattern

The milk and milk-rich beverages DP was significantly positively associated with spine T-score (r = 0.247, *P* = 0.008) and whole body BMD (r = 0.182, *P* = 0.051). However, this DP was not significant for hip and femoral neck T-score although a relatively high β-coefficient was recorded for femoral neck (r = 0.158, *P* = 0.099) and hip T-score (r = 0.148, *P* = 0.117) signifying a positive association (Table 6-4).

Table 6-4. Results of multiple linear regression of the dietary patterns (score values) and bone status

	Variance			
Parameters	**Explained (%)**	**β-coefficient**	**95% Confidence interval**	***P*-value**
Spine T-score	8.6			
Dietary pattern 1		0.247	0.094, 0.609	0.008
Dietary pattern 2		0.129	-0.071, 0.427	0.159
Dietary pattern 3		0.082	-0.138, 0.368	0.368
Dietary pattern 4		0.034	-0.202, 0.296	0.707
Femoral neck T-score	3.8			
Dietary pattern 1		0.158	-0.028, 0.316	0.099
Dietary pattern 2		0.0.16	-0.151, 0.178	0.869
Dietary pattern 3		0.034	-0.134, 0.194	0.719
Dietary pattern 4		-0.091	-0.354, 0.123	0.339
Hip T-score	3.0			
Dietary pattern 1		0.148	-0.038, 0.334	0.117
Dietary pattern 2		0.007	-0.173, 0.187	0.937
Dietary pattern 3		-0.015	-0.198, 0.168	0.873
Dietary pattern 4		-0.082	-0.260, 0.100	0.382
Whole body BMD	4.3			
Dietary pattern 1		0.182	-0.020, 0.020	0.051
Dietary pattern 2		0.009	0.009, 0.049	0.925
Dietary pattern 3		0.091	-0.033, 0.007	0.325
Dietary pattern 4		-0.038	-0.031, 0.010	0.681

Table 6-5 shows the multiple linear regression of the DPs against the body composition parameters. Of note is the significant negative relationship between the oily fish, sports drinks, seafood-rich DP and the total fat percentage. High negative correlations were also observed between waist circumference, BMI and DP 4 (Oily fish, sports drink and seafood-rich dietary pattern). For the waist circumference (r = -0.157, *P* = 0.094) and BMI (r = -0.163, *P* = 0.081) and DP 4, a negative association was also observed.

Table 6-5. Results of multiple linear regression of the dietary patterns (score values) and body composition

Parameters	Variance Explained (%)	β-coefficient	95% Confidence interval	*P*-value
Waist circumference	4.7			
Dietary pattern 1		-0.081	-3.034, 1.184	0.387
Dietary pattern 2		0.123	-0.676, 3.408	0.188
Dietary pattern 3		-0.043	-2.561, 1.586	0.642
Dietary pattern 4		-0.157	-3.780, 0.302	0.094
BMI	3.9			
Dietary pattern 1		0.032	-0.660, 0.937	0.731
Dietary pattern 2		0.100	-0.362, 1.235	0.281
Dietary pattern 3		-0.032	-0.938, 0.659	0.731
Dietary pattern 4		-0.163	-1.507, 0.090	0.081
Total fat percentage	6.3			
Dietary pattern 1		0.013	-1.142, 1.314	0.890
Dietary pattern 2		0.017	-1.116, 1.340	0.857
Dietary pattern 3		-0.034	-1.461, 0.995	0.708
Dietary pattern 4		-0.247	-2.901, -0.445	0.008

6.5 Discussion

The objective of the present research was to investigate the relationship between the dietary patterns generated from the FFQ provided by the women and their bone health status and body composition. The findings of this study showed that the high loading factor of milk and milk-rich beverages dietary pattern was positively correlated with spine T-score and whole-body BMD for the women. The dietary pattern reports a high loading of milks, milk-rich beverages such as coffee. This may probably be associated to the established coffee culture in New Zealand [16]. This milk and milk-rich beverages dietary pattern also had a high loading of fruit and yoghurt and cream.

The results of this study also highlighted that a dietary pattern characterised by high factor loadings of oily fish, sports drink and seafood and white fish was negatively associated with waist circumference, BMI and total body fat percentage. The intake of oily whole fish with bones such as sardine and tuna as well as seafood intake was negatively associated with waist circumference, BMI and total body fat percentage. It could be noted that the intake of sports drink which may signify a moderate-vigorous activity, was also negatively related to these body composition parameters.

Although the results of this study cannot be directly compared with that of other studies due to the differences in the protocols such as the number of food group classifications and food records, the dietary patterns generated are similar to those reported previously. This finding of a positive association between dairy and fruit, and bone health is in accordance with other studies that have reported a positive relationship between dairy and fruit dietary pattern and bone health [17-19]. The milk and milk-rich beverages dietary pattern in this study is similar to the dairy and fruit dietary pattern obtained by Shin and Joung 2013 from the Korean postmenopausal women [17]. Similarly, this dietary pattern can be compared to the ‘healthy’ dietary pattern obtained from Scottish postmenopausal women high in fruit, cheese, yoghurt/cream [19]. Of note is the reiteration that dairy foods is critical for bone health.

Calcium is important for bone metabolism and is a crucial component of the bone matrix [20]. Milk and dairy is an important source of calcium while dairy in combination with fruit intake has been reported as a good source of iron, vitamin A, C, K, thiamin, riboflavin, niacin and magnesium [21-23] linked to bone formation and bone health status mainly in post-menopausal women. The milk and milk-rich beverages dietary pattern would be of great benefit for bone health in post-menopausal women if improved simultaneously with vitamin D status [24].

The findings of this study also suggests that a dietary pattern with a high loading of oily fish, sports drink, seafood and white fish was negatively correlated with body composition most especially for total body fat percentage. The oily fish, sports drink and seafood dietary pattern which is similar to the Mediterranean diet was negatively correlated with total body fat percentage. The Mediterranean diet has been reported to be positively associated with body composition [25, 26]. However, sports drink featuring in this dietary pattern maybe as a result of the means by which the women replenish their electrolytes level during physical activity. The physically active women build lean body mass which has been previously positively linked to BMD [27].

The milk-rich beverage, coffee is a marker of high energy and as such body composition should be taken into account for bone health related studies. Moderate coffee intake (≤400mg/day of caffeine) appears to be potentially beneficial for metabolic health status [28]. On the other hand, coffee can also be considered as a dairy (milk) marker which could have been part of the patterning simply because milk is most often consumed with coffee.

The strengths of this study include the well-established data-driven statistical methodology and its high total variance explained by the four dietary patterns. The limitations of this study include its cross-sectional methodology and small sample size preventing causation.

6.6 Conclusion

The milk and milk-rich beverages dietary pattern was associated with spine T-score and whole body BMD and may therefore decrease the risks of osteoporosis in New Zealand postmenopausal women. This suggests that no matter how milk is consumed; the impact on bone health especially in postmenopausal women might be more important than originally thought.

This present findings also revealed that an oily fish, sports drink and seafood dietary pattern was negatively associated with total body fat percentage. This shows that this dietary pattern may have a positive influence for weight (fat) management in New Zealand postmenopausal women. However, further intervention research is warranted to investigate and confirm relationships between dietary patterns and hip and femoral neck T-scores in postmenopausal women.

Overall, the findings of this study suggest that there is an association between milk, milk-rich beverages and fruit intake and bone health. Likewise intake of fish (especially whole fish with bone such as sardine) and seafood may be beneficial for bone health in postmenopausal women.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Bolaji Lilian Ilesanmi-Oyelere
Name/title of Primary Supervisor:	Professor Marlena C. Kruger
Name of Research Output and full reference:	
Ilesanmi-Oyelere, B.L.; Schollum L.; Kuhn-Sherlock, B. et al. Inflammatory markers and bone health in postmenopausal women: a cross-sectional overview. Immunity and Ageing 2019	
In which Chapter is the Manuscript /Published work:	Chapter 7
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Chapter 7 Inflammatory markers and bone health in postmenopausal women: A cross-sectional overview

Nutrients make up our diet, and various energy-dense foods known as empty calorie foods such as foods high in fat and added sugar causes inflammation. The objective of this chapter was to determine levels of inflammatory markers in postmenopausal women and assess its association with bone status according to the WHO classifications of the T-scores at all sites, adiposity and ferritin levels.

This chapter is published as:

Ilesanmi-Oyelere, B.L.; Schollum L.; Kuhn-Sherlock, B. et al. Inflammatory markers and bone health in postmenopausal women: a cross-sectional overview. *Immunity and Ageing* 2019, 16:15.

7.1 Abstract

Cytokines, chemokines, C-reactive proteins (CRP) and ferritin are known inflammatory markers. However, cytokines such as interleukin (IL-1β), (IL-6) and tumour necrosis factor (TNF-α) have been reported to interfere with both the bone resorption and bone formation processes. Similarly, immune cell cytokines are known to contribute to inflammation of the adipose tissue especially with obesity. IL-10 but not IL-33 has been linked to lower ferritin levels and anemia. In this study, we hypothesized that specific cytokine levels in the plasma of women with low bone mineral density (BMD) would be higher than those in the plasma of healthy women due to the actions of elevated levels of pro-inflammatory cytokines in inducing osteoclast formation and differentiation during senescence.

Levels of cytokines (IFNα2, IFN-γ, IL-12p70, IL-33) and monocyte chemoattractant protein-1 (MCP-1) were significantly higher in the plasma of the osteoporotic group compared to the osteopenic and/or healthy groups. Meanwhile CRP levels were significantly lower in women with osteoporosis (P=0.040) than the osteopenic and healthy groups. Hip BMD values were significantly lower in women with high/detectable values of IL-1β (P=0.020) and IL-6 (P=0.030) compared to women where these were not detected. Similarly, women with high/detectable values of IL-1β had significantly lower spine BMD than those where IL-1β was not detected (P=0.030). Participants' CRP levels were significantly positively correlated with BMI, fat mass and fat percentage (P<0.001). In addition, ferritin levels of women with high/detectable values of anti-osteoclastogenic IL-10 (P=0.012) and IL-33 (P=0.017) were significantly lower than those where these were not detected. There was no statistically significant association between TNF-α and BMD of the hip and lumbar spine.

High levels of cytokines (IFNα2, IFN-γ, IL-12p70, IL-33) and MCP-1 in apparently healthy postmenopausal women are associated with low bone mass. In addition, an increase in levels of IL-10 and IL-33 may be associated with low ferritin levels in this age group.

7.2 Introduction

Cytokines are a large group of peptides and proteins, which are known to be involved in the signaling between the cells of the immune system [1, 2]. Examples of cytokines include interleukins (ILs), chemokines, interferons (IFNs), colony stimulating factors, tumour necrosis factors (TNFs), transforming growth factors (TGFs) and adipokines. Cytokines affect almost all biological processes in the body and can be either detrimental or beneficial depending on the amounts produced and conditions surrounding the production [1]. Cytokines play a critical role in the coordination of the immune system that is necessary for resolving bacterial and viral attacks on the immune system.

Furthermore, osteoporosis is a major public health concern which as a result of the demineralisation and weakening of bones leads to increased fracture risk [3]. Annually, reports suggest that osteoporosis causes more than 8.9 million fractures worldwide, resulting in an osteoporotic fracture every three seconds [4]. The burden of osteoporosis is therefore not limited to economic costs but also significant emotional and physical consequences, especially for middle aged and elderly men and women.

Aberrant or prolonged immune responses resulting in low-grade inflammation have been implicated in the pathogenesis of osteoporosis. In postmenopausal women, this is coupled with a decrease in oestrogen levels that leads to an increase in bone resorption [5]. Inflammation has also been related to indices of musculoskeletal health and several age-related diseases such as atherosclerosis, Alzheimer's disease and cancer [6].

C-reactive protein (CRP) is known to be a sensitive systemic inflammatory marker. The production of CRP in the liver, which upregulates levels of cytokines such as IL-1, IL-6 and TNF-α has been observed to be positively correlated with bone resorption including hip and spinal bone loss in healthy pre- and postmenopausal women [7-9].

Cytokines are also known as crucial regulators of the adipose tissue metabolism especially in obese individuals with body mass index (BMI) and fat percentage above 25kg/m^2 and 32% respectively as an indicator of obesity. Cell types, pre-adipocytes and mature adipocytes are able to promote secretion of cytokines and chemokines associated with increased mRNA expression, notably in obese individuals [10, 11]

Infection, injury or trauma influences iron status. Ferritin is known as an acute phase reactant, a marker of inflammation. In addition, serum ferritin concentration is well-known as an important indicator of total body iron stores. The hormone, hepcidin is a major regulator of systemic iron homeostasis in the liver and it is induced during inflammation leading to leakage of ferritin into the plasma from damaged cells. This causes sequestration of iron and increased serum ferritin.

Cytokines in general are often complicated to research due to their synergistic effects and their ability to affect or enhance each other's secretion. For example, IL-1 acts in synergy with TNF-α [12]. However, the cytokine network is significant in the regulation of the immune cells (primarily lymphocytes and macrophages) and the skeletal system where a natural balance is needed for bone metabolic homeostasis [13].

Based on observations and research in animal and *in-vitro* studies, cytokines have been classified according to their stimulatory or inhibitory effect on proliferation and differentiation of osteoclasts. Cytokines such as receptor activator of nuclear factor kappa-B ligand (RANKL), macrophage colony-stimulating factor (M-CSF), IL-1, IL-6, IL-7, IL-11, IL-15, IL-17, IL-23, IL-34, monocyte chemoattractant protein-1 (MCP-1), TNF-α, TNF-β have been reported for their stimulating effects on osteoclastogenesis (OC) [6, 14-18]. Meanwhile, IL-1ra, IL-3, IL-4, IL-10, IL-12, IL-18, IL-27, IL-33, interferon IFN-α, IFN-β, IFN-λ, OPG and transforming growth factor-β (TGF-β) have been reported to have inhibitory effect on the proliferation and differentiation of osteoclasts [6, 15, 19-22]. These studies have indicated that low-grade inflammation, due to the effect of pro-inflammatory cytokines, impairs DNA repair and leads to cellular and immunological senescence as well as biological ageing. Increase of IL-31 has also been linked to decreased BMD in postmenopausal women [23]. However, the course of inflammageing is multi-factorial, resulting not only from immunosenescence but also from several factors such as dietary patterns, obesity and gut microbiota status [6].

The aim of this study was to measure levels of inflammatory markers in postmenopausal women. The outcomes of this study were; 1. The relationship between levels of inflammatory markers and lumbar spine, hip BMD, bone markers and osteoporosis status. 2. The relationship between inflammatory markers and obesity/adiposity. 3. The relationship between anti-inflammatory cytokines and ferritin levels in apparently healthy postmenopausal women. To our knowledge, no study has related these 15 immune markers to bone health in postmenopausal women.

7.3 Methods

7.3.1 Subjects

A total of 127 New Zealand European postmenopausal women aged between 54 and 81 years participated in the "Bugs'n'Bones" study that took place in the Human Nutrition Research Unit at Massey University, Palmerston North campus from June to December 2017 [24]. Eighty-six women were then selected to participate in phase 2 for this study based on their bone strength for groups of healthy, osteopenic and osteoporotic women. Sample size was calculated using G*Power software version 3.0.10 and eighty-eight subjects were required for each group at a 90% power and an alpha of 5% for T-test. During this cross-sectional study, two subjects were excluded from the study, one due to consumption of a ketogenic diet and the other for health reasons. Subjects were recruited by advertisement on campus, the Wanganui Chronicle and by using a recruitment agency; Trial Facts (https://trialfacts.com/). The inclusion criteria were menopause of at least 5 years based on no menstruation.

Exclusion criteria were presence of any systemic disease, food intolerances affecting the gastrointestinal tract, smoking and high intake of alcohol. Subjects with significant weight loss or weight gain within the past year were also excluded. All subjects were free living and apparently healthy. Written informed consent was obtained from subjects before commencing data collection.

7.3.2 Anthropometric and body composition measurements of the subjects

The body weight of subjects was measured using the Detecto 437 eye-level weigh beam physician scale to the nearest 0.1kg and standing height was measured using a stadiometer to the nearest 0.1cm wearing light clothes and no shoes. Body mass Index (BMI) was calculated as weight divided by height squared (kg/m^2) using the Quetelet's index. Waist to hip ratio was determined by measuring the waist and hip circumference to the nearest 0.1cm using a non-stretchable measuring tape. Waist to hip ratio was calculated as a marker of abdominal obesity.

Body composition measurements including fat mass (FM), lean mass (LM) and fat percentage were measured and analysed using the Hologic QDR series Discovery A, Bone densitometry system [Dual energy X-ray Absorptiometry (DXA)]. Bone mineral density was measured at the femoral neck (FN), lumbar spine (LS) [L1-L4], trochanter, Ward's triangle and total hip. The *in vivo* reproducibility of the coefficient of variation ranged between 0.34 - 0.70% for all measured sites. The DXA machine was calibrated every morning for all the measurements and at the end of each day. The reported spine BMD values were calculated as means of four measured values from L1–L4. Apex System Software version 4.5.3 was used to analyse the DXA scans. Osteoporosis was defined as a T score ≤ -2·5 and osteopenia as T score between -1.0 and -2.5 according to the WHO criteria [25].

7.3.3 Biochemical blood and bone marker collection and analyses

Plasma samples were collected from centrifuged fasting venous blood samples and stored frozen at -80^0C before analysis. Cytokine assays were prepared using BioLegend® LEGENDplex™ Multi-Analyte Flow Assay kit's instructions and measured using the Beckman Coulter's Gallios flow cytometer. Levels of 13 cytokines, namely IL-1β, IFN-α2, IFN-λ, TNF-α, MCP-1, IL-6, IL-8, IL-10, IL-12p70, IL-17A, IL-18, IL-23 and IL-33 were quantified in plasma from the subjects. LEGENDplex™ Data Analysis Software version 8.0 was used to analyze the data. Plasma levels of CRP and ferritin were measured using the electrochemiluminescence immunoassay "ECLIA".

Bone markers C-terminal telopetide (CTX-1) and total procollagen type 1 N-terminal propeptide (P1NP) were analysed by electrochemiluminescence immunoassay using the Roche COBAS® e411 system (Roche Diagnostics, Indianapolis, IN, USA), while vitamin 25(OH)D_3 was analysed using isotope-dilution liquid chromatography-tandem mass spectrometry (ID-LC-MS-MS) by Canterbury Health, Christchurch, New Zealand.

7.3.4 Statistical analysis

IBM SPSS version 25 (IBM Company, Armonk, NY, USA) and Minitab were used for the tabular representation of data while R statistical software and Excel were used for the graphical representations. The outcome variables used were spine and hip BMD and T-score. The values of all variables are presented as mean ± standard deviation. One-way analysis of variance (ANOVA) was used to compare the mean values of the CRP quartiles with the subjects' characteristics. Likewise, ANOVA was used for the comparison of the means of osteoporosis classes (healthy, osteopenia and osteoporosis groups) against levels of cytokines. The mean difference between grouped IL-1β and IL-6 levels and the subjects' characteristics was determined using the independent t-test.

Pearson correlations were used to investigate the relationship between inflammatory markers (continuous) and physiological parameters and bone markers. Partial correlation analyses of the inflammation markers (adjusted for age, height and BMI) were performed against lumbar spine and hip bone mineral content (BMC), BMD, T-score and the bone markers. Correlation results were presented as Pearson correlation coefficient (r) and p-value. All p-values were reported significant at 0.05 or less.

7.4 Results

The characteristics of the participants who were included in the study are shown in Table 7-1. Mean (SD) age for participants was 63.2 (4.6) years. The BMI, calculated using the Quetelet's index, ranged from 14.9 to 38.3 kg/m^2.

Table 7-1 Characteristics of participants

Parameters (n=86)	**Mean± SD**	**Min**	**Max**
Age (years)	63.2±4.6	54	81
Weight (kg)	67.3±10.2	43.0	89.2
Height (cm)	161.9±5.2	149.1	173.5
BMI (kg/m^2)	25.7±3.8	14.9	38.3
WC (cm)	79.7±10.9	57.0	110.0
HC (cm)	98.0±7.0	78.0	112.0
WH Ratio	0.8±0.1	0.7	1.0
Spine BMC	53.3±11.6	31.3	82.6
Spine BMD	0.9±0.2	0.5	1.3
Spine T-score	-1.1±1.4	-4.6	2.6
Hip BMC	29.2±5.2	19.0	44.2
Hip BMD	0.8±0.1	0.6	1.1
Hip T-score	-0.8±0.9	-2.5	1.4
Total Fat%	40.4±6.0	14.8	51.1
Total lean mass (kg)	40.1±4.5	30.7	51.2
Ferritin (µg/L)	142.9±100.9	15.00	467.00
25(0H)D_3 (nmol/L)	78.1±23.5	21.00	160.00

BMI=body mass index; WC=waist circumference; HC=hip circumference; WH=waist to hip; BMC=bone mineral content; BMD=bone mineral density

Subsequently, one-way ANOVA was performed to compare the mean differences according to the quartiles of CRP levels (Table 7-2). Significant increase in BMI and various body fat percentage was observed as CRP levels increased indicating a positive correlation. On the other hand, the relationship between BMD and T-score with CRP levels was inconsistent and not statistically significant.

Table 7-2 Characteristics according to quartiles of CRP

Parameters	**<0.60mg/L (n=26)**	**0.61-1.0mg/L (n=17)**	**1.01-2.20mg/L (n=20)**	**>2.21mg/L (n=20)**	**F-value**	***P*-value**
Age (years)	62.4(4.1)	64.5(6.4)	63.9(4.3)	63.1(3.9)	0.871	0.460
BMI (kg/m^2)	23.3(3.4)	25.7(3.4)	27.6(2.6)	27.2(4.3)	7.489	<0.001
WC (cm)	73.3(9.7)	80.6(12.0)	84.7(7.2)	83.0(11.3)	5.853	0.001
Waist to Hip ratio	0.77(0.07)	0.82(0.09)	0.84(0.06)	0.83(0.09)	4.150	0.009
Spine BMD	0.89(0.18)	0.91(0.14)	0.93(0.15)	0.94(0.16)	0.434	0.729
Spine T-score	-1.4(1.6)	-1.2(1.2)	-1.1(1.4)	-0.9(1.3)	0.533	0.661
Hip BMD	0.81(0.12)	0.86(0.12)	0.84(0.12)	0.85(0.08)	0.715	0.546
Hip T-score	-1.27(0.88)	-0.55(1.00)	-0.68(1.06)	-0.73(0.67)	0.795	0.500
Android fat %	32.3(8.1)	35.0(8.6)	39.9(5.9)	39.3(8.2)	4.795	0.004
Gynoid fat %	40.7(7.3)	43.2(4.0)	44.6(3.6)	44.6(4.4)	2.881	0.041
Android:Gynoid ratio	0.79(0.13)	0.81(0.17)	0.90(0.12)	0.87(0.14)	2.737	0.049
Trunk fat %	32.8(7.1)	36.4(7.3)	40.4(4.2)	40.2(7.5)	6.778	<0.001
Body fat %	36.9(6.9)	40.2(5.7)	42.9(2.9)	42.9(5.6)	6.072	0.001
TFM/TLM	0.6(0.2)	0.7(0.2)	0.8(0.1)	0.8(0.2)	7.094	0.001
Ferritin (μg/L)	134.0(118.9)	160.4(105.8)	130.5(89.5)	154.1(93.0)	0.398	0.755
25(OH)D_3 (nmol/L)	83.6(24.8)	80.0(16.6)	75.1(23.6)	73.2(24.8)	0.955	0.418

BMI=body mass index; WC=waist circumference; BMD=bone mineral density; TFM=total fat mass; TLM=total lean mass; CRP=C-reactive protein. Variables are reported as mean values with their standard deviation (SD). Number of participants (n).

The relationship between levels of inflammatory markers and osteoporosis status of the participants was also investigated (Table 7-3). The spine classification was used because of a higher incidence of osteoporosis at the spine than the hip.

Mean levels of all the inflammatory markers were higher among the osteoporotic group except in the case of CRP, which was significantly lower in the osteoporotic women, and TNF-α and IL-6 levels which did not show consistent patterns. Meanwhile, there were significant differences for IFN-α2 (P=0.027), IFN-γ (P=0.009), MCP-1 (P=0.055), IL-12p70 (P=0.049), and IL-33 (P=0.048) across the groups.

Table 7-3 Cytokines/inflammatory markers according to spine osteoporosis status

Markers	Osteoporotic (n=13)	Osteopenic (n=34)	Healthy (n=39)	F-value	*P*-value
IL-1β (pg/ml)	7.22(17.59)	2.06(4.88)	1.52(4.21)	2.670	0.075
IFN-α2 (pg/ml)	35.28(48.19)	9.96(18.95)	18.42(26.63)	3.783	0.027
IFN-γ (pg/ml)	153.38(222.32)	50.67(58.72)	57.91(73.24)	4.956	0.009
TNF-α (pg/ml)	2.01(2.70)	1.83(3.72)	3.19(6.02)	0.795	0.455
MCP-1 (pg/ml)	221.36(100.46)	176.66(43.13)	200.73(55.68)	3.000	0.055
IL-6 (pg/ml)	3.22(4.29)	3.14(11.26)	3.09(10.61)	0.001	0.999
IL-8 (pg/ml)	23.85(42.34)	11.19(11.84)	10.66(14.99)	2.220	0.115
IL-10 (pg/ml)	4.62(15.67)	0.57(1.52)	0.75(1.95)	2.261	0.111
IL-12p70 (pg/ml)	6.47(17.03)	1.86(5.74)	0.48(1.36)	3.128	0.049
IL-17A (pg/ml)	102.40(227.62)	41.75(79.92)	31.11(45.72)	2.303	0.106
IL-18 (pg/ml)	153.45(72.70)	133.21(69.18)	144.98(51.66)	0.600	0.551
IL-23 (pg/ml)	103.40(173.69)	56.60(91.14)	42.84(55.70)	1.970	0.146
IL-33 (pg/ml)	13.88(37.44)	0.79(3.32)	2.83(11.19)	3.147	0.048
CRP (µg/ml)	0.75(1.07)	1.39(1.36)	2.04(2.05)	3.219	0.044
Ferritin (µg/L)	149.46(116.16)	124.45(94.57)	156.29(103.72)	0.890	0.415

IL=interleukin; IFN=interferon; MCP=monocyte chemoattractant protein; CRP=C-reactive protein. Osteoporosis status was according to WHO classification[25]. Variables are reported as mean values with their standard deviation (SD). Number of participants (n).

Based on the hip osteoporosis classification; ANOVA results of MCP-1 were significantly higher in the osteoporotic group compared to the osteopenic and healthy group (p=0.003), however it should be noted that the osteoporotic group was small (n = 13).

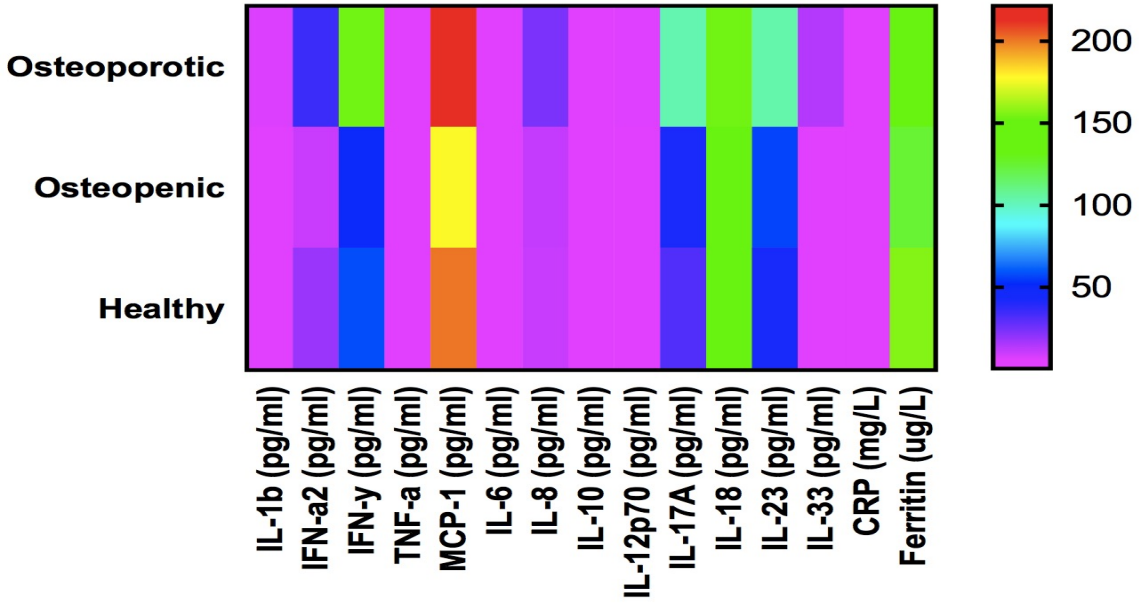

Figure 7-1 **Heat map of inflammatory markers by spine osteoporosis classification**

Figure 7-1 shows the heat map of inflammatory markers by spine osteoporosis status. Red colour denotes high (>200) while the purple colour signifies low (<50) units of the inflammatory markers. The cytokines and chemokines were generally higher in the osteoporotic group than the healthy/normal or the osteopenic groups in this apparently healthy postmenopausal women (Fig. 7-1).

Investigating the relationship between IL-1β and bone status further, significant differences were found between categories of IL-1β (<2.39pg/ml and >2.39pg/ml) and spine BMC, BMD, T-score and hip BMD as shown in Table 7-4. Higher levels of IL-1β were associated with lower BMC, BMD and T-score.

Table 7-4 Independent T-test of characteristics of participants by IL-1β

Variables	**IL-1β (<2.39pg/ml) [68]**	**IL-1β (>2.39pg/ml) [18]**	***P*-value**
Age (years)	63.29±4.58	62.89±4.86	0.742
Weight (kg)	67.31±10.28	67.37±10.25	0.981
Height (cm)	161.65±5.19	162.57±5.20	0.506
BMI (kg/m^2)	25.77±3.87	25.48±3.62	0.770
WC (cm)	79.79±10.92	79.37±11.10	0.885
Waist-Hip ratio	0.81±0.08	0.81±0.08	0.990
Spine BMD	0.94±0.16	0.85±0.14	0.028
Spine T-score	-0.94±1.43	-1.77±1.26	0.021
Femoral Neck BMD	0.71±0.10	0.66±0.08	0.019
Hip BMD	0.85±0.12	0.79±0.09	0.020
Hip T-score	-0.73±0.95	-1.16±0.86	0.071
Lean mass (kg)	40.07±4.43	40.37±4.78	0.798
Total Fat%	40.34±6.22	40.40±5.36	0.967
Ferritin (µg/L)	145.03±107.45	134.28±81.40	0.647
25(OH)D_3 (nmol/L)	78.32±29.94	77.00±18.62	0.806

IL=interleukin; BMI=body mass index; WC=waist circumference; BMC=bone mineral content; BMD=bone mineral density; CRP=C-reactive protein. Variables are reported as mean values with their standard deviation (SD). Number of participants [n].

Table 7-5 shows the relationship between IL-6 and participants' characteristics indicating significant differences between IL-6 and weight, waist circumference as well as the hip BMC and BMD. There were higher levels of IL-6 found in individuals with lower BMC, BMDs and T-scores.

Table 7-5 Independent T-test of participants' characteristics by IL-6

Variables	**IL-6 (<1.62pg/ml) [53]**	**IL-6 (>1.62pg/ml) [33]**	***P*-value**
Age (years)	63.34±4.86	63.00±4.24	0.734
Weight (kg)	65.66±10.05	70.00±10.03	0.054
Height (cm)	161.66±5.01	162.15±5.49	0.672
BMI (kg/m^2)	25.09±3.47	26.69±4.16	0.057
WC (cm)	77.59±9.77	83.11±11.88	0.021
Waist-Hip ratio	0.80±0.07	0.84±0.09	0.020
Spine BMD	0.94±0.15	0.90±0.17	0.343
Spine T-score	-0.99±1.37	-1.32±1.52	0.291
Femoral Neck BMD	0.71±0.10	0.67±0.09	0.035
Hip BMD	0.86±0.12	0.81±0.10	0.033
Hip T-score	-0.64±0.98	-1.10±0.82	0.023
Lean mass (kg)	39.55±4.59	41.07±4.18	0.117
Total Fat%	39.55±6.37	41.65±5.25	0.100
Ferritin (µg/L)	142.75±107.04	143.12±91.74	0.987
25(OH)D_3 (nmol/L)	80.17±24.14	74.85±22.46	0.303

IL=interleukin; BMI=body mass index; WC=waist circumference; BMC=bone mineral content; BMD=bone mineral density; CRP=C-reactive protein. Variables are reported as mean values with their standard deviation (SD). Number of participants [n].

Table 7-6 shows the correlation coefficients of the inflammatory markers against bone health indicators. Significant negative correlations were found between IL-12p70 and bone health parameters as well as between CRP and the bone turnover markers P1NP and CTX-1. Furthermore, in Table 7-6 vitamin 25(OH)D_3 was most often significantly positively correlated with inflammatory markers.

Table 7-6 Correlations of inflammatory markers with bone health indicators across groups

	Spine BMD	Spine T-score	Hip BMD	Hip T-score	P1NP (ug/L)	CTX-1 (ug/L)	PTH (pmol/L)	25(OH)D_3 (nmol/L)
IL-1β (pg/ml)	-0.211*	-0.216*	-0.181	-0.154	0.010	-0.049	-0.027	0.092
IFN-α2 (pg/ml)	-0.065	-0.076	-0.130	-0.138	-0.154	-0.226*	0.062	0.051
IFN-γ (pg/ml)	-0.106	-0.115	-0.181	-0.175	-0.125	-0.189	-0.005	0.218*
TNF-α (pg/ml)	0.153	0.151	0.075	0.068	-0.020	-0.003	0.068	-0.067
MCP-1 (pg/ml)	0.049	0.035	-0.024	-0.023	-0.117	-0.124	0.013	0.287**
IL-6 (pg/ml)	-0.018	-0.036	-0.089	-0.094	-0.207	-0.145	-0.033	0.127
IL-8 (pg/ml)	-0.120	-0.128	-0.164	-0.155	0.039	-0.049	-0.105	0.209
IL-10 (pg/ml)	-0.172	-0.177	-0.157	-0.164	-0.051	-0.142	-0.104	0.185
IL-12p70 (pg/ml)	-0.220*	-0.227*	-0.241*	-0.250*	-0.103	-0.192	-0.148	0.195
IL-17A (pg/ml)	-0.182	-0.189	-0.123	-0.092	-0.009	-0.091	-0.180	0.245*
IL-18 (pg/ml)	0.023	0.029	-0.069	-0.075	-0.160	-0.143	-0.002	0.097
IL-23 (pg/ml)	-0.176	-0.185	-0.131	-0.090	-0.026	-0.046	-0.073	0.246*
IL-33 (pg/ml)	-0.098	-0.102	-0.161	-0.167	-0.026	-0.087	-0.074	0.234*
CRP (μg/ml)	0.190	0.196	0.148	0.164	-0.253*	-0.223*	0.132	-0.132
Ferritin (μg/L)	0.190	0.228*	0.178	0.197	0.080	0.064	-0.156	0.024

* $p < .05$, **$p < .01$. IL=interleukin; IFN=interferon; TNF=tumour necrosis factor; MCP=monocyte chemoattractant protein; CRP=C-reactive protein; BMD=bone mineral density.

Partial correlations adjusted for the effect of age and BMI on the relationship between inflammatory markers and bone health were explored in Table 7-7. IL-6 was significantly negatively correlated with hip BMD, hip T-score and P1NP. Vitamin 25(OH)D_3 was positively correlated with MCP-1, IL-6, IL-17A and IL-23.,

Table 7-7 Partial correlations of inflammatory markers with bone health indicators adjusting for age and BMI

	Spine BMD	Spine T-score	Hip BMD	Hip T-score	P1NP (ug/L)	CTX-1 (ug/L)	PTH (pmol/L)	25(OH) D_3 (nmol/L)
IL-1β (pg/ml)	-0.188	-0.197	-0.217	-0.188	0.010	-0.064	-0.039	0.092
IFN-α2 (pg/ml)	-0.003	-0.017	-0.075	-0.087	-0.185	-0.222*	0.065	0.054
IFN-γ (pg/ml)	0.020	0.008	-0.103	-0.101	-0.145	-0.221*	0.028	0.174
TNF-α (pg/ml)	0.183	0.180	0.102	0.092	-0.009	-0.012	0.064	-0.079
MCP-1 (pg/ml)	0.084	0.067	0.024	0.027	-0.086	-0.154	0.037	0.277**
IL-6 (pg/ml)	-0.105	-0.131	-0.256*	-0.266*	-0.222*	-0.166	-0.107	0.228*
IL-8 (pg/ml)	0.015	0.005	-0.093	-0.087	0.004	-0.081	-0.093	0.190
IL-10 (pg/ml)	-0.100	-0.108	-0.160	-0.174	-0.069	-0.163	-0.115	0.190
IL-12p70 (pg/ml)	-0.094	-0.102	-0.185	-0.202	-0.156	-0.228*	-0.128	0.158
IL-17A (pg/ml)	-0.057	-0.067	-0.049	-0.011	-0.062	-0.123	-0.167	0.240*
IL-18 (pg/ml)	0.109	0.117	-0.016	-0.025	-0.163	-0.169	-0.007	0.124
IL-23 (pg/ml)	-0.051	-0.062	-0.039	0.010	-0.079	-0.076	-0.050	0.232*
IL-33 (pg/ml)	0.016	0.011	-0.096	-0.108	-0.039	-0.102	-0.053	0.205
CRP (μg/ml)	0.004	0.009	-0.077	-0.053	-0.257*	-0.256*	0.072	-0.030
Ferritin (μg/L)	0.112	0.157	0.136	0.160	0.093	0.058	-0.191	0.049

* $p < .05$, **$p < .01$. IL=interleukin; IFN=interferon; TNF=tumour necrosis factor; MCP=monocyte chemoattractant protein; CRP=C-reactive protein; BMD=bone mineral density.

Furthermore, as shown in Table 7-8, partial correlations adjusted for age and height (to reflect BMC which varies with height) indicated stronger relationships between the 15 markers of inflammation and bone, especially negative correlations between IL-1β and IL-12p70 and spine BMC/spine BMD/spine T-score. In addition, vitamin 25(OH)D_3 was positively correlated with IFN-γ, MCP-1, IL-8, IL-10, IL-12p70, IL-17A, IL-23 and IL-33.

Table 7-8 Partial correlations of inflammatory markers with bone health indicators adjusting for age and height

	Spine BMC	Spine BMD	Spine T-score	Hip BMC	Hip BMD	Hip T-score	P1NP (ug/L)	CTX-1 (ug/L)	PTH (pmol/L)	25(OH)D_3 (nmol/L)
IL-1β (pg/ml)	-0.224*	-0.221*	-0.229*	-0.209	-0.264*	-0.238*	0.000	-0.079	-0.054	0.106
IFN-α2 (pg/ml)	-0.015	-0.031	-0.044	-0.072	-0.109	-0.121	-0.198	-0.238*	0.057	0.058
IFN-γ (pg/ml)	-0.111	-0.117	-0.129	-0.203	-0.260*	-0.258*	-0.158	-0.245*	-0.020	0.220*
TNF-α (pg/ml)	0.182	0.157	0.154	0.073	0.050	0.039	-0.047	-0.050	0.073	-0.155
MCP-1 (pg/ml)	0.004	0.022	0.005	-0.015	-0.072	-0.070	-0.125	-0.200	0.029	0.267*
IL-6 (pg/ml)	-0.120	0.000	-0.022	-0.075	-0.123	-0.133	-0.222*	-0.156	-0.067	0.165
IL-8 (pg/ml)	-0.089	-0.118	-0.128	-0.194	-0.258*	-0.253*	-0.024	-0.121	-0.131	0.223*
IL-10 (pg/ml)	-0.173	-0.166	-0.174	-0.229*	-0.232*	-0.244*	-0.067	-0.169	-0.141	0.225*
IL-12p70 (pg/ml)	-0.257*	-0.226*	-0.234*	-0.338**	-0.331**	-0.344**	-0.152	-0.236*	-0.173	0.222*
IL-17A (pg/ml)	-0.197	-0.188	-0.198	-0.248*	-0.220	-0.186	-0.082	-0.156	-0.205	0.278*
IL-18 (pg/ml)	0.087	0.040	0.046	-0.019	-0.116	-0.125	-0.205	-0.216	-0.018	0.117
IL-23 (pg/ml)	-0.193	-0.178	-0.190	-0.248*	-0.217	-0.172	-0.117	-0.125	-0.087	0.257*
IL-33 (pg/ml)	-0.094	-0.096	-0.101	-0.187	-0.213	-0.223*	-0.039	-0.112	-0.092	0.253*
CRP (µg/ml)	0.142	0.171	0.177	0.061	0.137	0.157	-0.233*	-0.216	0.127	-0.105
Ferritin (µg/L)	0.182	0.182	0.223*	0.261*	0.197	0.218	0.066	0.041	-0.145	-0.027

* $p < .05$, **$p < .01$. IL=interleukin; IFN=interferon; TNF=tumour necrosis factor; MCP=monocyte chemoattractant protein; CRP=C-reactive protein; BMC=bone mineral content; BMD=bone mineral density.

Figure 7-2 shows the Pearson correlations of inflammatory markers against CTX-1 when separated into the osteoporotic, osteopenic, healthy and across all groups. There is a significant positive correlation between IFN-α2 and CTX-1 amongst the osteoporotic group, and a negative correlation between CRP and CTX-1 in the healthy group.

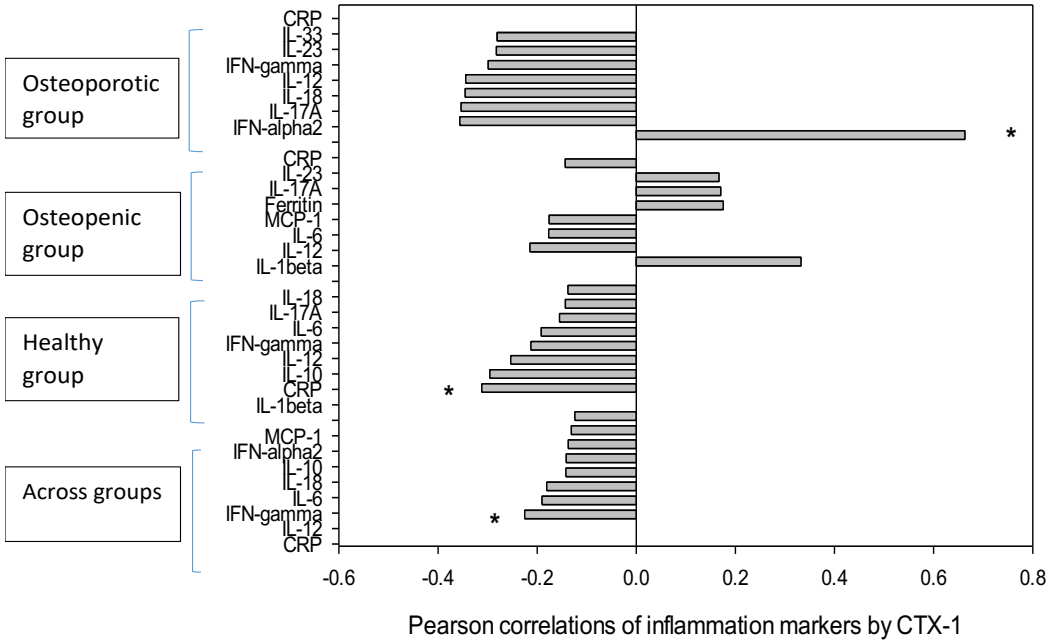

Figure 7-2 **Pearson correlations of inflammatory markers by CTX-1**

7.4.1 The relationship between inflammatory markers and fat percentage across groups

The bar graphs in Fig. 7-3 show the correlations between the 15 inflammatory markers and fat percentage. Levels of CRP were the most strongly positively correlated with whole body fat percentage (WBFatp), as oppose to IL-23 which was strongly negatively correlated.

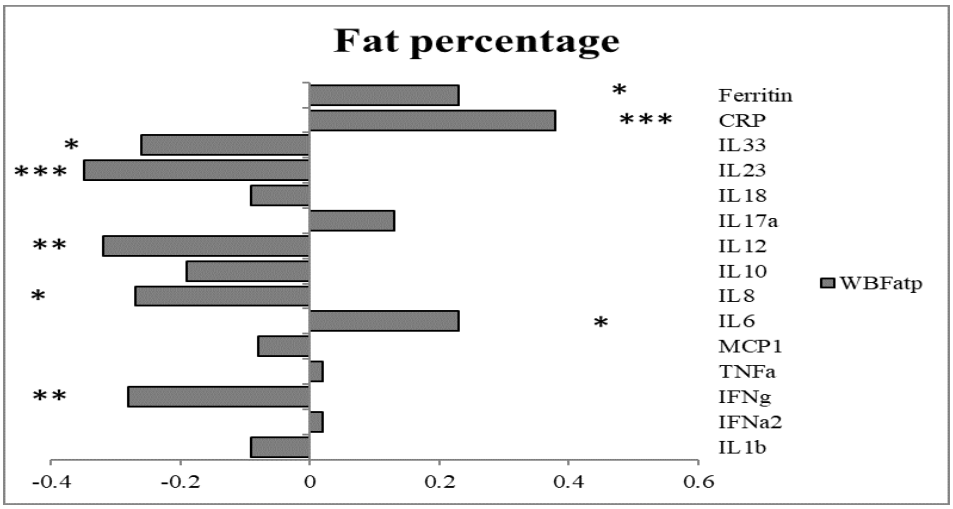

Figure 7-3 **Correlations of inflammation markers by fat percentage**

∗ p < .05, ∗∗p < .01, ∗∗∗ p < .001

From Fig. 7-3, we observed significant positive correlations between ferritin, CRP and IL-6 and the fat percentage. All other cytokines were negatively correlated with the fat percentage, with statistically significant correlations for IL-33, IL-23, IL-12, IL-8 and IFN-γ.

7.4.2 Ferritin as a marker of inflammation

Analysis of anti-inflammatory cytokines' production against ferritin levels indicated significant differences between IL-10 (p-value = 0.012), IL-33 (p-value = 0.017) and the levels of ferritin. High levels of ferritin were significantly associated with low levels of IL-10 and IL-33, indicating a possible anti-inflammatory effect of IL-10 and IL-33 on the ferritin status of individuals.

In summary, these results provide important insights into a cross-sectional overview of inflammatory markers and bone health during midlife and senescence. Thereby, providing platforms for further hypotheses and research in this area.

7.5 Discussion

In general terms, the cytokines measured in this study can be described as

Inflammatory (osteoclastogenic) cytokines include IL-1β, IL-6, IL-8, IL-17, MCP-1, TNFα, IFN-α2 and IFN-γ). They are generally known for their degenerating and catabolic effects on tissue metabolism and homeostasis as well as the intracellular actions and signalling pathway to osteoclastic differentiation [26]. More so, in postmenopausal women, when coupled with the effect of oestrogen deficiency. Elevated levels of inflammatory cytokines have been linked with lower bone mineral density [12], as part of "inflammaging" [27].
Anti-Inflammatory (anti-osteoclastogenic) cytokines include IL-10, IL-18, IL-33. Most often anti-inflammatory cytokines exert opposite effects to those of inflammatory cytokines on bone.
Dual-role cytokines include IL-12 and IL-23. In certain circumstances, these cytokines provide a balancing act, with a dual-role in the regulation of the immune system.
It must be appreciated that although individual cytokines may be described as pro- or anti-inflammatory, they interact closely with one another as part of a dynamic network which creates a balance of both inhibitory and stimulatory immune effects [26].

Some studies, although limited and contradictory, have investigated the impact of immune cytokines, especially IL-6, on bone loss in postmenopausal women [28-32]. However, to our knowledge no study has investigated the relationship of all the 15 immune markers with bone health in postmenopausal women.

7.5.1 CRP

CRP is a sensitive marker of systemic inflammation. The production of CRP in the liver upregulates other inflammatory cytokines (including IL-1, IL-6 and TNFα) and has been shown to be positively correlated with bone resorption and hip and spinal bone loss in healthy pre- and post-menopausal women [7-9].

In our study CRP was significantly associated with measures of BMI and various measures of body fat. High BMI, body fat and fat percentage was associated with high CRP, supporting previous studies linking obesity with increased systemic inflammation [33]. These results are similar to those of the Dunedin study that reported a positive correlation between CRP and BMI [34]. Similar results to our findings was also reported by Berglundh et al. 2015, their results indicated that women with lower CRP quartiles had lower BMD values [8].

However, in contrast to the literature, CRP was lower in the osteoporotic women than those with osteopenia or healthy bone. This may reflect a lower body weight in women with osteoporosis or may be an effect of the small number of women with osteoporosis in this study. In addition, CRP was negatively correlated with both P1NP (a bone formation marker) and CTX-1 (a bone resorption marker), indicating a lower overall bone turnover with high levels of CRP. However, the findings of this study is in accordance to that of Huang and Schooling which found no association between higher hsCRP and lower BMD [35].

Of interest also was the observation that higher levels of CRP were associated with low vitamin 25(OH)D_3 levels. Although this relationship did not reach statistical significance it is potentially worth investigating further in future studies.

7.5.2 Immune Cytokines and Bone Health

With participants grouped into healthy, osteopenic and osteoporotic groups based on spine BMD measurements, (Table 3) the means of all immune markers (with the exception of CRP, discussed above) were higher in the osteoporotic group, and ANOVA demonstrated significant differences for IFN-α2, INF-γ, IL-12P70, IL-33, and trending increased in IL-1β, MCP-1, and IL-23. This increase in both pro- and anti-inflammatory markers may indicate a more active upregulated immune status in those with poor bone health.

Unexpectedly, we observed higher levels of IL-10 (an osteoprotective cytokine) in the osteoporotic group and correlations with bone health indicators were negative. In addition, although IL-33 has been reported as an anti-osteoclastogenic cytokine [6, 36], we found levels of IL-33 to be significantly higher in the osteoporotic group than the normal or osteopenia groups, and correlations with bone health parameters were negative although they did not reach statistical significance. The negative correlations between IL-33 and bone resorption marker CTX as reported by Ginaldi et al. (2019) is however similar to the result of this study [36]. However, further studies with larger numbers of women are needed in this area.

In agreement with the literature [29, 37, 38], independent T tests showed a significant negative relationship between IL-1β and bone parameters – high pro-inflammatory IL-1β was associated with lower BMC, BMD and T-scores (Table 4). For proinflammatory IL-6, there was a positive association with BMI, WC and waist-hip ratio, and a negative association with femoral neck BMD, hip BMD and hip T-score.

7.5.3 Correlations

Analysis of correlations between bone parameters and immune markers (Table 6) showed a significant negative correlation between IL-12p70 and spine and hip bone measurements. Adjustment for age and height retained this relationship, as well as showing a significant negative relationship between IL-1β and spine and hip bone measurements. The other associations with hip measurements are not discussed further due to low numbers of women with poor hip bone health. Additional data are needed to confirm these associations.

Of note from these correlations are the associations of immune markers with bone biomarkers: IFN-α2, IFN-γ, and IL-12p70 had a negative association with CTX-I, so high levels of potentially inflammatory cytokines were associated with low CTX-1 and hence low bone resorption activity. Similarly, IL-6 showed a negative correlation with P1NP, so high levels of pro-inflammatory IL-6 were associated with low levels of bone formation. Evidence for the anti-inflammatory effects of vitamin 25(OH)D_3 were observed in its correlations with the inflammatory markers. Vitamin 25(OH)D_3 was positively correlated with all the inflammatory cytokines except TNF-α and CRP.

7.5.4 Inflammatory markers and body fat percentage

Body fat and obesity have been reported to be positively associated with low-grade inflammation [33, 39]. This was further illustrated in our study, where BMI and various body fat measurements (including fat mass, fat percentage, abdominal fat, waist circumference, waist to hip ratio, android fat, gynoid fat, android to gynoid fat ratio, trunk fat percentage, and body fat percentage, and fat mass to lean mass ratio) all had significant positive correlations with CRP and IL-6 (both markers of inflammation).

In addition, we found significant negative correlations between several of the potentially anti-inflammatory markers (IL-23, IL33, and IL-12) and body fat percentage in all groups of women. The proinflammatory markers IL-8 and IFN-γ also had negative correlations with body fat percentage, which may reflect the complex cytokine interactions regulating/balancing the immune system. In general, high body fat percentage indicating obesity appears to be associated with increased inflammation and lower levels of anti-inflammatory cytokines.

7.5.5 Ferritin and anti-inflammatory cytokines

Infection, injury or trauma influences iron status. However, iron in the body is stored as ferritin in normal healthy individuals. Serum ferritin concentration is well-known as an important, convenient and accurate indicator of total body iron stores in humans. Hepcidin hormone is a major regulator of systemic iron homeostasis in the liver and it is induced during inflammation. Thus, ferritin is also known as an acute phase reactant, a marker of inflammation.

Hypoferremia and a high serum ferritin concentration during acute phase response has been linked to the actions of pro-inflammatory cytokines both *in vivo* and *in vitro* [40]. High levels of ferritin have been suggested to act as a potent oxidant causing oxidative stress which is associated with increased risk of various diseases [41]. Thus, iron levels must be regulated and controlled in order to provide an essential nutrient that is capable of oxygen delivery and metabolism with regulated redox reactions, but without causing cellular damage or apoptosis by guarding against excessive toxicities [42, 43].

In the liver, during infection with extracellular bacteria, hepcidin targets ferroportin (FP1) [a transmembrane protein that transports iron from the inside of a cell to the outside] and results in its degradation which causes a reduction in hepcidin levels produced by macrophages as a result of inflammatory cytokines; giving rise to hypoferremia (reduced iron availability for extracellular bacteria) and high levels of ferritin [44].

In an experimental study by Feelder et al., following an administration of inflammatory cytokines (TNF-α, IL-1 and IL-6), an increase in ferritin synthesis and a decrease in serum iron were observed [40]. Furthermore, Nairz et al. reported that in response to the actions of pro-inflammatory cytokines such as IL-6 and IFN-γ, systemic hypoferremia and increased ferritin were observed as a result of extracellular actions [44].

Furthermore, ferritin has been postulated as a disease marker and mediator [45]. However, contrary to our findings, ferritin has been suggested to be responsible for immune suppression via increased production of IL-10 in the presence of chemokines [45].

The results from our cross-sectional study showed that anti-inflammatory cytokines IL-10 and IL-33 are significantly related to lower plasma ferritin status in a cohort of post-menopausal women. This suggests a possible role of anti-inflammatory, anti-osteoclastogenic cytokines in iron metabolism and the regulation of iron stores. Further research work is needed to confirm the role of anti-inflammatory cytokines in iron homeostasis.

Overall, our findings showed that in a group of apparently healthy postmenopausal women, cytokine levels of IFNα2, IFN-γ, IL-12p70, IL-33 and MCP-1 were significantly higher in groups with low bone mass (osteoporosis) than those with higher bone mass (osteopenia and healthy). We also found that when converted into categorical data, there were significant differences in levels of IL-1β according to the spine and hip classifications. Similarly, we observed significant differences in levels of IL-6 by the hip categorical classification. Partial correlation coefficients with adjustment for age and height showed negative correlations between IL-1β, IFN-α2, IFN-γ, IL-6, IL-8, IL-10, IL-12p70, IL-23, IL-33 and hip and spine BMC, BMDs, and T-scores. Furthermore, we found that high levels of ferritin were significantly associated with low IL-10 and IL-33 levels in these postmenopausal women.

Limitations of this study include the cross-sectional nature preventing any cause and effect relationship and the small number of osteoporotic postmenopausal women compared to the healthy and osteopenic groups.

7.6 Conclusion

In summary, although specific individual cytokines were correlated with bone health (most clearly demonstrated by the observation that high levels of IL-β were associated with low spine and hip bone measurements), many cytokines (both pro- and anti-inflammatory) were shown to be elevated in osteoporotic women, perhaps indicating upregulation of the overall immune system in women with poor bone health.

Some inflammatory markers were also shown to have an impact on bone turnover: CRP was negatively correlated with both P1NP (a bone formation marker) and CTX-1 (a bone resorption marker), indicating lower overall bone turnover with high levels of CRP. High levels of potentially inflammatory cytokines (IFN-α2, IFN-γ, and IL-12p70) were associated with low CTX-1 and hence low bone resorption activity, and high levels of pro-inflammatory IL-6 were associated with low levels of the bone formation marker P1NP. Of note, high fat percentage indicating obesity was associated with increased inflammation and lower levels of anti-inflammatory cytokines.

This study strengthens the knowledge of the role of immune factors in promoting bone degradation in older women. Despite these promising results, to develop a full picture of the impact of cytokines and chemokines on bone health, additional longitudinal intervention studies are recommended to confirm and expand the relationships described in this study.

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STATEMENT OF CONTRIBUTION
DOCTORATE WITH PUBLICATIONS/MANUSCRIPTS

We, the candidate and the candidate's Primary Supervisor, certify that all co-authors have consented to their work being included in the thesis and they have accepted the candidate's contribution as indicated below in the *Statement of Originality*.

Name of candidate:	Bolaji Lilian Ilesanmi-Oyelere
Name/title of Primary Supervisor:	Professor Marlena C. Kruger
Name of Research Output and full reference:	
Ilesanmi-Oyelere, B.L.; Rafiadal, E.; Roy, N.; Coad, J.; and Kruger, M.C. Gut microbiota composition, diversity and predictive function in healthy and osteoporotic postmenopausal women	
In which Chapter is the Manuscript /Published work:	Chapter 8
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Chapter 8 Gut microbiota composition, diversity and predictive function in healthy and osteopenic/osteoporotic postmenopausal women

The objective was to determine whether there are differences in the microbiota diversity between the healthy (H) and osteopenic/osteoporotic (OP) postmenopausal groups of women as classified using the WHO bone density classification. The associations between gut microbiota composition and BMD sites were investigated. This chapter also presents the gene abundance of gut microbes and associated predictive functional pathways that may be important for postmenopausal bone health.

This chapter is for submission to Journal of Bone Mineral Research

8.1 Abstract

The gut microbiota has been postulated to affect nutrient absorption, the endocrine and immune systems and bone metabolism. The gut microbiota, otherwise known as the largest gene pool of the human body may play a significant role in bone health. One hundred and twenty-seven postmenopausal women were recruited from the Manawatu-Whanganui region, for a cross-sectional study and 86 participated in the phase II trial which involved faecal sample collection. The women were classified into two groups of healthy (H) and osteopenic/osteoporotic (OP) based on the WHO classification of their BMD and resultant T-scores. Faecal DNA samples collected from eligible post-menopausal women underwent whole genomic sequencing by Illumina HiSeq® 2500 System. The microbial composition diversity (alpha diversity based on Shannon index) was significantly decreased among the OP group compared to the healthy group when using the hip classification ($P_{Shannon}$ = 0.013) as well as femoral neck ($P_{Shannon}$ = 0.0003) status but not for the spine ($P_{Shannon}$ = 0.40) classification. The composition (beta diversity by Bray-Curtis index) did not differ significantly between the groups based on the hip T-score (P < 0.081) and the spine T-score (P < 0.692) but was significant based on the femoral neck T-score (P < 0.009) status. The phylum *Bacteroidetes* were negatively correlated with the BMD sites, while the *Firmicutes* (most *Lactobacillus* and *Bacillus*) were positively correlated with the BMD sites. The interferon gamma receptor 1 (CD119) K05132 orthology group was significantly more abundant for the osteopenic/osteoporotic group based on the hip and FN osteoporosis classification. This orthology group is linked to the cytokine-cytokine receptor interaction, HIF-1 signaling pathway, necroptosis, osteoclast differentiation, JAK-STAT signaling pathway, Th1 and Th2 as well as Th17 cell differentiation, all of which has been linked to skeletal muscle degeneration and ultimately osteoporosis. The results presented here provide a possible link between certain members of the gut microbiota and bone loss/ osteoporosis. These findings provide a basis for a longitudinal intervention to assess the role of the gut microbiome in prevention of osteoporosis.

8.2 Introduction

The gut microbiota, now referred to as a novel 'endocrine organ' is capable of secreting hormones or hormone-like substances for regulating host hormone levels and further influencing host health status. The gut microbiota produce oestrogen-like metabolites which have been proven to exert regulatory effects on bone metabolism [1]. This is particularly important in postmenopausal osteoporosis because of oestrogen deficiency and ageing, where an increase in bone loss is pronounced. The rapid decline in circulating oestrogen levels has been correlated with expansion of Th17 cells (osteoclastogenic population of CD4+ T cells) and increased serum levels of pro-inflammatory cytokines, including tumor necrosis factor α (TNFα), interleukin-1β (IL-1β), IL-8, IL-17 and IL-6, as well as the osteoclastogenic cytokine receptor activator of nuclear factor-κB ligand (RANKL) [2]. Maintaining the homeostasis of the gut microbiota is therefore critical to bone health due to the observed relationship between the gut microbiota and bone metabolism.

Three possible mechanisms of the gut microbiota-bone health relationship have been proposed based on previous studies, performed mainly in animal models [3]. First, the immune-mediated regulation of bone metabolism through the control of osteoclastogenic cytokine production and $CD4^+$ T cell production has been documented [2]. A study showed that absence of gut microbiota in germ-free mice led to decreased osteoclast precursor cells, reduced pro-inflammatory cytokines and lesser CD4+ T cells in the bone and bone marrow compared to the control mice raised under conventional conditions [4]. These studies suggest that the gut microbiota may play a role in the regulation of bone metabolism through the immune system [5, 6]. The second mechanism could be the endocrine-mediated regulation of bone metabolism via the production of growth hormones through the insulin growth factor (IGF-1) signalling pathway [7], serotonin [8] and gonadal steroids [9]. The third pathway involves the gut microbiota regulating bone metabolism through the enhancement of calcium absorption [1, 10]. Also, bone health maintenance can be achieved through the role of the gut microbiota in improving calcium balance via the transcellular pathway in the small intestine and paracellular pathway in the large intestine as well as its effect on the lumen pH [1].

The gut microbiota is thought to modulate the bone metabolism by inducing or producing metabolites and fermentative substrates such as short chain fatty acids (SCFA), mainly acetate, butyrate and propionate [11]. These fermentative substrates can be generated from prebiotics in the diet and endogenously, thereby lowering the pH of the lumen to inhibit formation of calcium complexes such as calcium phosphate and consequently increasing the absorption of calcium [1].

Probiotics, prebiotics and synbiotics (containing probiotics and their favourable prebiotics) increase calcium bioavailability in adults by enhancing its absorption [1, 12] while most probiotics and commensal microbes are known to ferment prebiotics to produce the fermentative substrates mentioned above. Beneficial effects of probiotics such as *Lactobacillus* species and *Baccillus clausii* as well as prebiotics such as inulin and galacto-oligosaccharides (GOS) on bone formation have been recorded in animal models however robust data from human studies are still lacking [2]. To date and to our best knowledge, there are two human studies that have investigated the role of gut microbiota in bone health. The two studies used 16S ribosomal RNA (rRNA) gene sequencing to characterise the microbiota composition profile [13, 14]. Wang et al. (2017) found the relative abundance of *Blautia*, *Parabacteroides* and *Ruminococcaceae* been higher in the osteoporotic patients than the normal control [13]. This study was conducted with a small sample size of mainly female patients recruited from a Chinese hospital with mean age between 64 - 70 years. Das et al. (2019) found that *Lactobacillus* was proportionally more abundant in participants with osteoporosis compared to those with normal BMD [14]. This study involved 181 female and males aged between 55 - 75 years care referrals. Limited published epidemiological evidence supports the relationship between the gut microbiota and bone mineral density and robust human studies are required to evaluate this relationship.

We hypothesised that the healthy (H) postmenopausal women will have a higher microbial diversity and community composition than postmenopausal women with osteopenia/osteoporosis (OP). Shotgun metagenomics sequencing was used to analyse the composition profile of the microbiota using faecal samples of 86 postmenopausal women classified as either H or OP groups based on the T-scores at various skeletal sites. The faecal samples were used as a proxy of the microbiota composition in the large intestine. The DNA community composition, diversity and predictive functionality (based on microbial gene abundance) were compared between H and OP groups based on the hip, femoral neck and spine bone density and WHO T-score classifications.

8.3 Materials and Methods

8.3.1 Subjects

Eighty-six postmenopausal women aged 54 - 81 years participated in the phase II of the "Bugs'n'Bones" study that took place in the Human Nutrition unit of Massey University, Palmerston North between October 17th, 2017 and March 6th, 2018. A total of 39 women were healthy and 47 osteopenic or osteoporotic based on the spine T-score status whereas 47 were healthy and 39 osteopenic or osteoporotic based on the hip T-score status. Furthermore, 27 participants were healthy while 59 were osteopenic or osteoporotic based on the femoral neck status. The groups were then classified into either H or OP (for those with osteopenia and osteoporosis) groups.

The inclusion criteria were confirmed as menopause of at least 5 years based on no menstruation. Exclusion criteria were presence of any systemic disease, food intolerances which affects the gut, smokers and high intake of alcohol (>2 units per day) or use of antibiotics within 3 months of the study. Participants with significant weight loss or weight gain within the past year were excluded. Written informed consent was obtained from participants before commencing data collection. The study was registered with the Australian New Zealand Clinical Trials Registry (ANZCTR) with the number ACTRN12617000802303. This study was approved by Massey University Human Ethics Committee: Southern A, Application 17/17.

8.3.2 Anthropometric and body composition measurements of the participants

Body weight of women was measured using the Detecto 437 eye-level weigh beam physician scale to the nearest 0.1kg. Standing height was measured using a stadiometer to the nearest 0.1cm wearing light clothes and no shoes on. The BMI was calculated as weight divided by height squared (kg/m^2). Waist to hip ratio was determined by measuring the waist and hip circumference to the nearest 0.1cm using a non-stretchable measuring tape. Waist to hip ratio was calculated as a marker of abdominal obesity.

Body composition measurements, fat mass, lean mass and fat percentage were measured and analysed using the Hologic QDR series Discovery A, Bone densitometry system [Dual energy X-ray Absorptiometry (DXA)]. BMD was measured at the femoral neck (FN), lumbar spine (LS) [L1-L4], trochanter, Ward's triangle and total hip. The DXA machine was calibrated every morning for all the measurements and at the end of each day. The reported BMD values were calculated as means of four measured values from L1–L4. Apex System Software version 4.5.3 was used for analysing the DXA scans.

Osteoporosis was defined as LS T score ≤ 2·5 and osteopenia as LS T score between -1.0 and -2.5 according to the WHO criteria [15]. Participants were classified into two groups of either H or OP women based on the spine, hip and FN T-scores.

8.3.3 Sample collection and DNA Isolation

Participants were provided with a faecal sample collection kit and were instructed to collect faeces into a container in an anaerobic bag with an anaerobic sachet and freezer pack and brought to the Human Nutrition unit within 2-3 hours of collection according to standard practice [16]. Faecal samples were then stored at -80ºC.

DNA samples were extracted from the faecal samples with Bioline Isolate Feacal DNA kit (Bioline, Australia) as per manufacturer's instruction within one week of sample collection and stored at -80ºC for about five months before sequencing. The purity and concentration of DNA samples were tested using a Nanodrop 2000 Spectrophotometer (Thermo Fisher Scientific, USA).

8.3.4 Library preparation, microbiota sequencing and quality control

The library preparation was conducted by Massey Genome Service (MGS) using the Nextera XT DNA library prep kit (Illumina, USA). DNA was then sent to Otago Genomics and Bioinformatics Facility (OGBF) for shotgun genomic sequencing by using the HiSeq®2500 System guide (Illumina, USA). Raw reads were filtered to remove low quality reads.

8.3.5 Microbial community analyses

Alpha diversity estimates were conducted for within-sample richness and evenness by the Shannon diversity index. Richness refers to the quantity of different species present in the community. Beta diversity was computed for between-sample dissimilarity. Multivariate statistics were conducted for the analysis of similarity (ANOSIM), Adonis test and "betadisper" for the analysis of group dispersion (Appendix 7). The permutational multivariate analysis of variance, PERMANOVA test was performed by using the Adonis test. The robust principal coordinate analysis (PCoA) was performed with Bray-Curtis index. All taxonomic analyses were conducted using the web-based interface Microbiome Analyst.

8.3.6 Statistical analyses

Independent-samples *t* test was used to compare the differences between variables that conform to normal distribution while Mann-Whitney *U* test was used for those that did not conform to normal distribution between groups. Values were expressed as mean ± standard deviation. Spearman's correlation was conducted due to some of the variables not conforming to normal distribution. Relative abundance for each sample was generated based on the RefSeq data from all microbes.

8.3.7 Analyses of functional profiles

Functional profiles were analysed using the web-based interface Microbiome Analyst for comprehensive visualisation and meta-analysis of the gut microbiome data generated. (https://www.microbiomeanalyst.ca/MicrobiomeAnalyst/faces/home.xhtml) Low count filter was 20% prevalence in sample. Low variance filter was based on inter-quantile range. Cumulative sum scaling was used for data scaling. The data was not rarefied. Table 8-1 shows the data type and data properties.

Table 8-1 Table of functional analyses profile

Data type:	Gene abundance table
File format:	text
Gene annotation:	ko
Total gene number:	274
Genes with ≥ 2 counts:	262
Number of experimental factors:	36
Total read counts:	201962524
Average counts per sample:	2348401
Maximum counts per sample:	3758275
Minimum counts per sample:	1474044
Phylogenetic tree uploaded:	No
Number of samples in metadata:	86
Number of samples in OTU table:	86
Sample names match (metadata vs. OTU table):	Yes
Number of sample names matched (metadata vs. OTU table):	86
Number of samples that will be processed:	86

8.4 Results

8.4.1 Participants' characteristics

The age of the participants ranged from 54 – 81 years old. The healthy individuals had higher BMD and T-scores based at the hip, FN and spine compared to the OP groups (P<0.001) as shown in Table 8-2.

Table 8-2 Characteristics of participants classified according to the spine osteoporotic status

Parameters	Healthy (H)[39]	Osteoporotic (OP)[47]	P-value
Age (years)	63.15±5.25	63.26±4.06	0.920
BMI	27.44±3.47	24.26±3.48	<0.001
WC	84.49±10.04	75.74±10.04	<0.001
Waist-Hip ratio	0.84±0.08	0.79±0.07	0.007
Spine T-score	0.18±0.92	-2.19±0.69	<0.001
Spine BMD	1.07±0.10	0.80±0.08	<0.001
Total hip T-score	-0.21±0.85	-1.32±0.68	<0.001
Total hip BMD	0.92±0.10	0.78±0.07	<0.001
Femoral neck T-score	-0.82±0.80	-1.82±0.55	<0.001
Femoral neck BMD	0.76±0.09	0.65±0.06	<0.001

BMI=body mass index; WC=waist circumference; BMD=bone mineral density

8.4.2 Gut microbial composition differences between healthy and osteoporotic groups

A total of 1,040,278,291 read counts were generated from the 86 DNA samples from the participants with an average of 12,096,259 counts per sample. In total, the faecal DNA microbial samples consisted of 49 phyla, 106 classes, 192 orders, 351 families, 744 genera. A total of 1279 OTU and 1132 OTU with ≥ 2 counts were quantified from the data.

Microbial diversity (alpha diversity based on both richness (OTU count) and evenness of the community) was significantly decreased among the OP group compared to the healthy women based on the hip T-score WHO classification status ($P_{Shannon}$ = 0.013), FN T-score status ($P_{Shannon}$ = 0.0003) but not significant for the spine T-score status ($P_{Shannon}$ = 0.402) (Fig. 8-1 to 8-3). However, the diversity of the communities based on only the richness was not significant.

A.

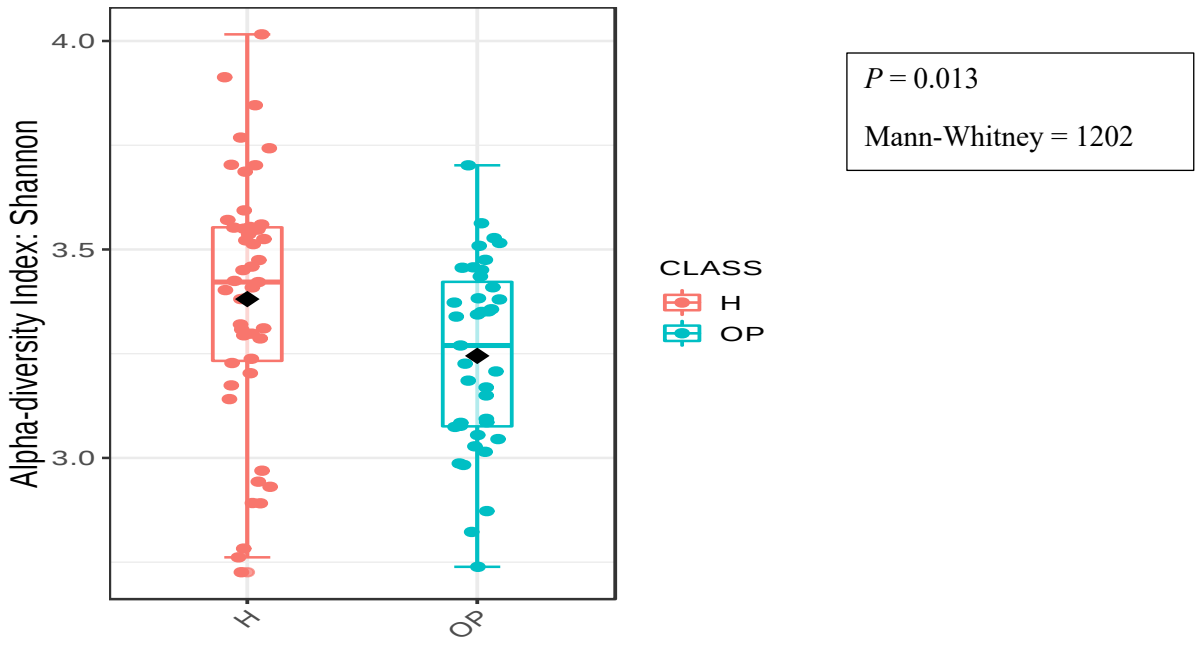

Figure 8-1 **Alpha diversity of faecal DNA samples by Shannon diversity. (A) – Classification of groups according to the hip BMD and T-score**

B.

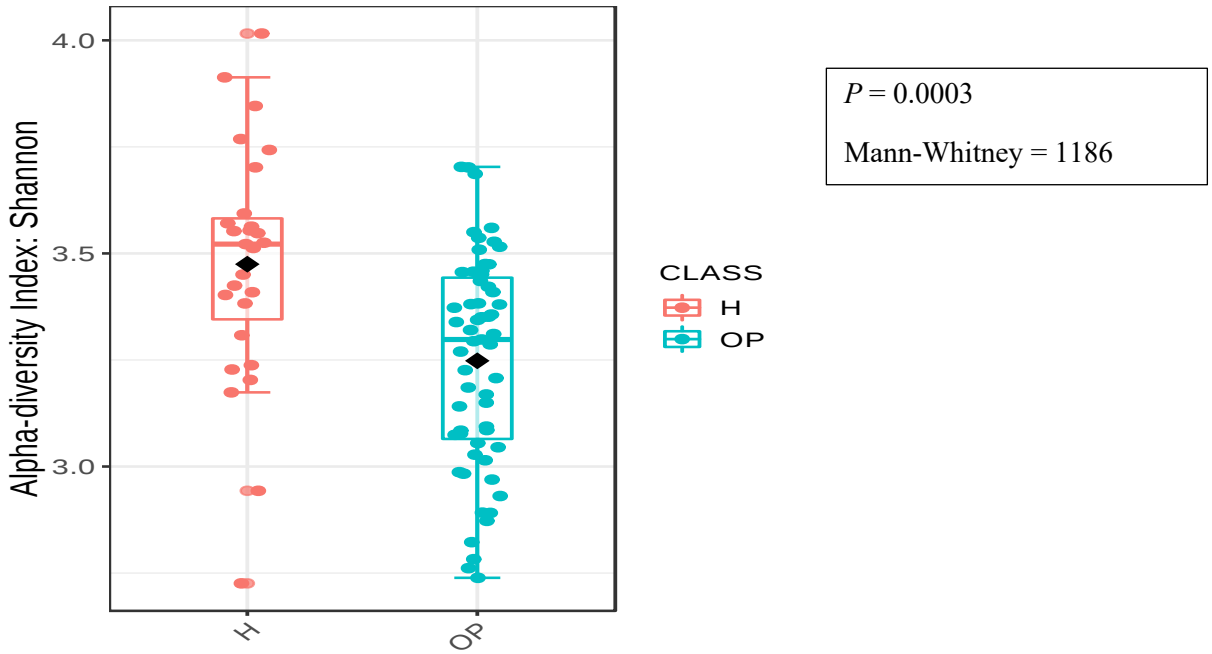

Figure 8-2 **Alpha diversity of faecal DNA samples by Shannon diversity. (B) – Classification of groups according to the femoral neck BMD and T-scores**

C.

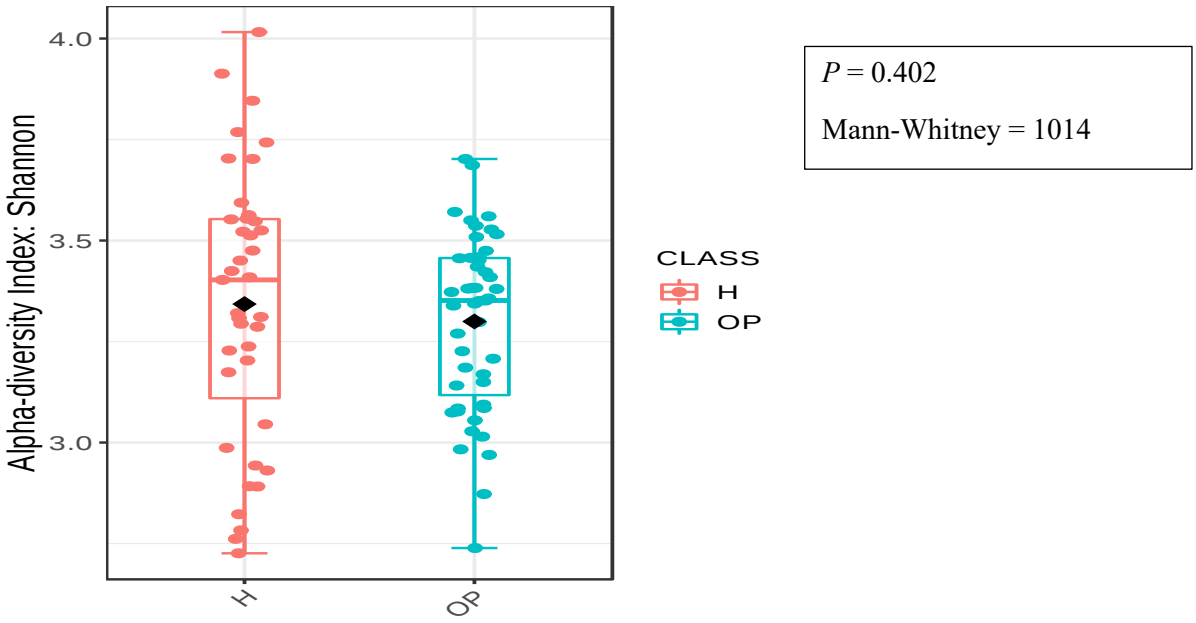

Figure 8-3 **Alpha diversity of faecal DNA samples by Shannon diversity. (C) Classification of groups according to the spine BMD and T-scores. H = healthy, OP = osteopenic/osteoporotic.**

The microbial composition (beta diversity) did not differ significantly between the groups based on the hip status (P_{ANOSIM} = 0.394, P_{ADONIS} = 0.066, $P_{betadisper}$ = 0.066) but was significant for the FN (P_{ANOSIM} = 0.032, P_{ADONIS} = 0.007, $P_{betadisper}$ = 0.775) and the spine (P_{ANOSIM} = 0.041, P_{ADONIS} = 0.440, $P_{betadisper}$ = 0.010) (Appendix 8). The PCoA also known as the metric multidimensional scaling produces an ordination based on a distance or dissimilarity matrix using the Bray-Curtis Index (Fig. 8-4-8-6).

The species diversity based on the Shannon index shows how different and how the microbes are balanced to each other. This indicates how the microbes are evenly distributed in a sample. As can be observed from the results; there is a higher microbial alpha diversity for the healthy in comparison to the OP group for the hip, femoral neck and spine BMD/T-score osteoporosis classifications. Furthermore, beta diversity analysis was conducted to investigate the dissimilarity between the communities.

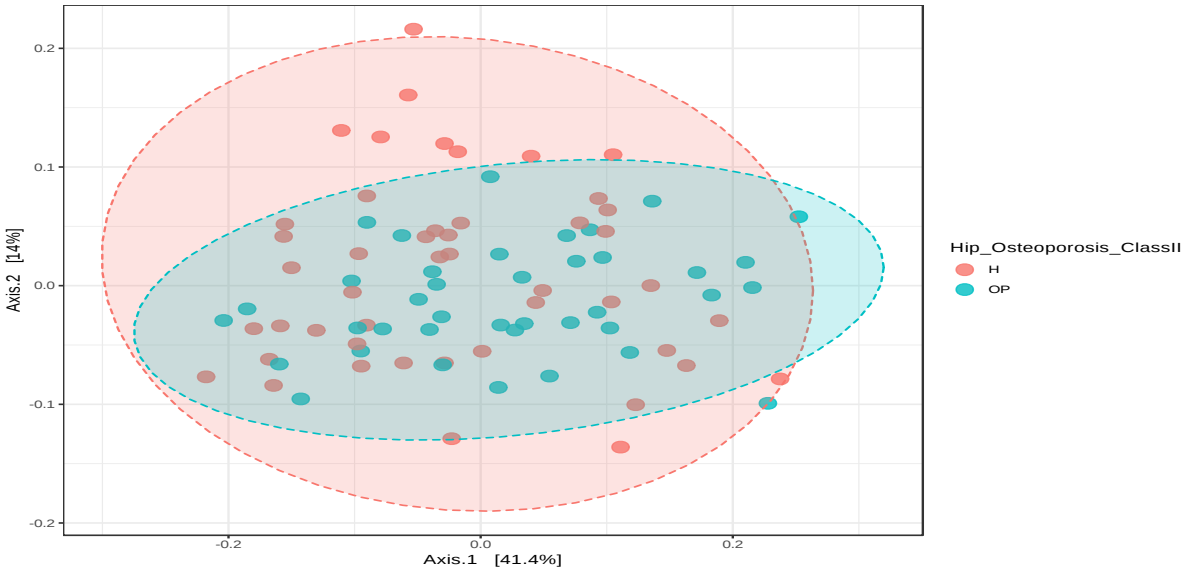

Figure 8-4 **Principal coordinate analysis plots by Bray-Curtis index for samples according to the hip T-score classification. [Permanova] F-value:1.9397; R^2: 0.02257; p value < 0.081**

H – healthy and OP – osteopenic/osteoporotic according to the hip T-score status.

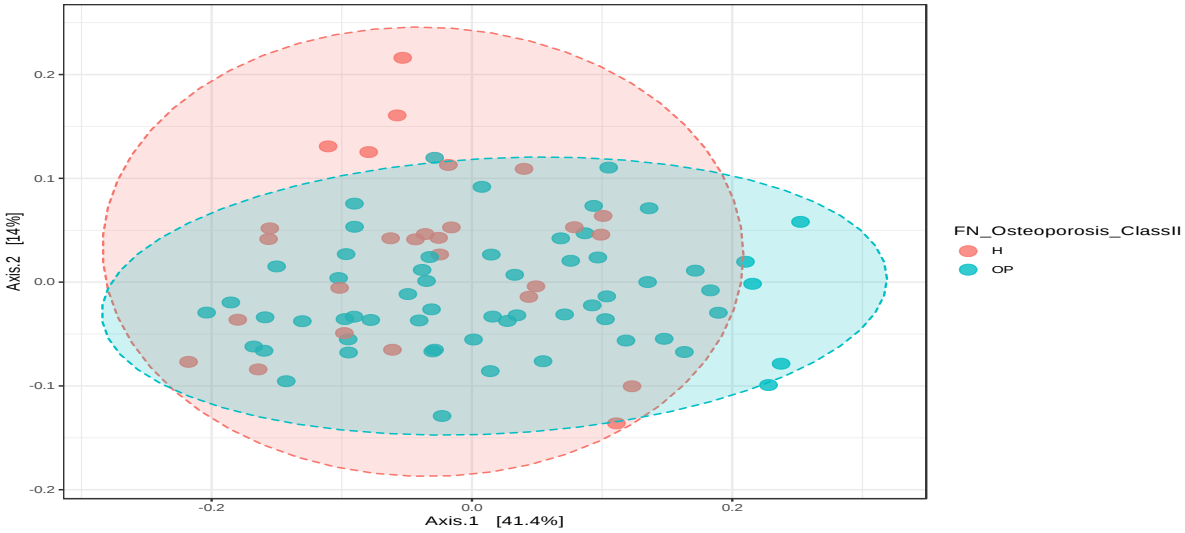

Figure 8-5 **Principal coordinate analysis plots by Bray-Curtis index for samples according to the femoral neck T-score classification. [Permanova] F-value: 3.568; R^2:0.040745; p value < 0.009**

H – healthy and OP – osteopenic/osteoporotic according to the femoral neck (FN) T-score status.

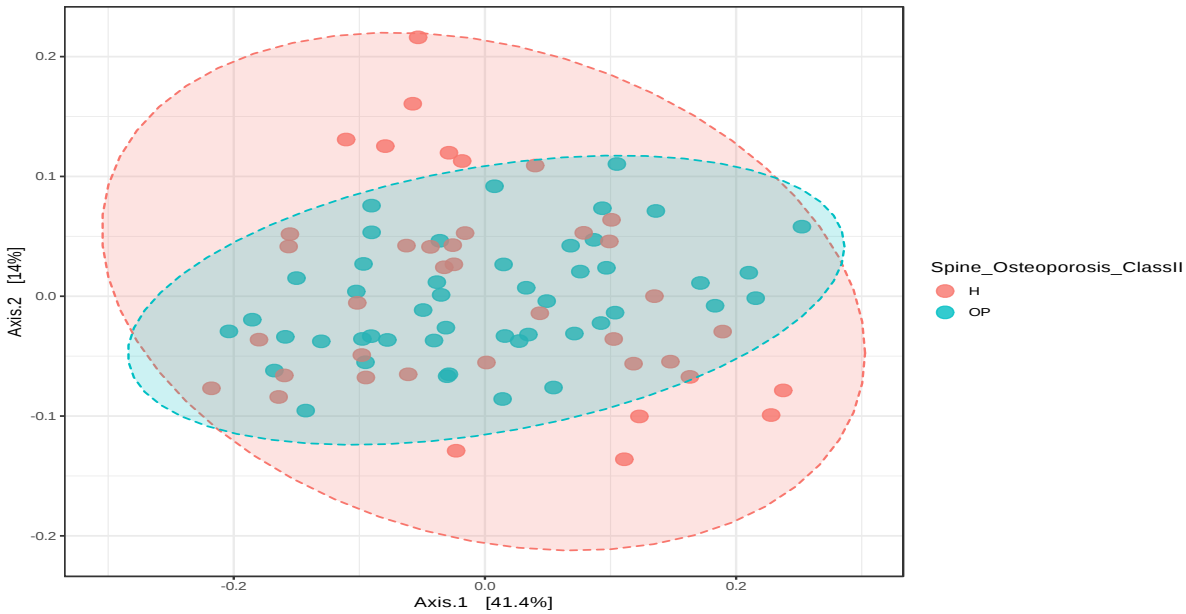

Figure 8-6 **Principal coordinate analysis plots by Bray-Curtis index for samples according to the spine T-score classification. [Permanova] F-value: 0.63673; R^2:0.0075231; p value < 0.692**

H – healthy and OP – osteopenic/osteoporotic according to the spine T-score status.

The Bray-Curtis index was utilised in order to gauge the dissimilarity and diversity of the community sample sets of H versus OP women. In Fig. 8-4 to 8-6, the principal coordinate analyses are shown. These figures show that axis 1 accounts for 41.4% variation while axis 2 accounts for 14%. Though R^2 for the graphs were low and not significant for Fig. 8-4 and 8-6, however, Fig. 8-5 shows there was a significant difference between the groups. In addition, Spearman correlations was then performed in order to identify how the microbes was correlated with the bone sites.

8.4.3 Correlations

Results of the Spearman correlations between relevant microbes and spine, hip, FN and whole-body BMD values showed significant positive correlations between *Lactobacillus* and BMD at all sites. However, *Bacteroides* and *Parabacteroides* were negatively correlated with BMD values at all sites (Table 8-3). Interestingly, *Bacteroidetes Alistipes* (known for digesting fats) was positively associated with the BMD but this was not significant.

The *Bacteroidetes Bacteroides* was most abundant for the OP groups when assessed with the BMD data at all sites compared to the H groups (Appendix 9). Positive correlations were observed between *Lactobacillus, Bacillus, Paenibacillus* and BMD at all sites. Of note, is the positive correlation between *Alkaliphilus*, an alkaliphilic bacterium and BMD.

Table 8-3 Spearman correlations of gut microbiota OTU abundances with BMD measures

	BMD sites			
Taxonomic level	**Spine**	**Hip**	**FN**	**WB**
p__Firmicutes__g__Bacillus_123	0.13	0.30***	0.43***	0.20
p__Firmicutes__g__Lactobacillus_585	0.22*	0.25*	0.35***	0.28**
p__Verrucomicrobia__g__Akkermansia_44	0.22*	0.088	0.14	0.23*
p__Firmicutes__g__Roseburia_995	-0.081	-0.092	-0.13	-0.12
p__Fusobacteria__g__Fusobacterium_457	0.059	0.22*	0.33***	0.14
p__Actinobacteria__g__Bifidobacterium_146	0.017	-0.006	0.073	0.10
p__Bacteroidetes__g__Bacteroides_124	-0.12	-0.27*	-0.33***	-0.17
p__Spirochaetes__g__Brachyspira_162	0.057	0.25*	0.29**	0.19
p__Euryarchaeota__g__Methanobrevibacter_666	0.14	0.17	0.29**	0.007
p__Firmicutes__f__Ruminococcaceae_1270	0.088	0.23*	0.26*	0.18
p__Firmicutes__g__Enterococcus_409	0.12	0.26*	0.26*	0.24*
p__Bacteroidetes__g__Parabacteroides_831	-0.17	-0.24*	-0.22	-0.16
p__Firmicutes__f__Lachnospiraceae_1253	-0.024	0.036	0.041	0.01
p__Firmicutes__g__Clostridium_286	-0.047	0.14	0.21	0.04
p__Bacteroidetes__g__Alistipes_51	0.077	0.065	0.055	0.12
p__Firmicutes__g__Caldicellulosiruptor_187	0.10	0.29**	0.37***	0.25*
p__Firmicutes__g__Ethanoligenens_429	0.12	0.29**	0.33***	0.30**
p__Firmicutes__g__Alkaliphilus_53	0.13	0.32***	0.42***	0.24*
p__Firmicutes__g__Geobacillus_467	0.12	0.29**	0.42***	0.21*
p__Firmicutes__g__Paenibacillus_824	0.15	0.33***	0.41***	0.22*
p__Firmicutes__g__Caldanaerobacter_186	0.10	0.28**	0.37***	0.22*
p__Firmicutes__g__Thermoanaerobacter_1134	0.08	0.26**	0.35***	0.21*
p__Firmicutes__g__Desulfitobacterium_356	0.13	0.31**	0.36***	0.23*

BMD=bone mineral density; FN=femoral neck; WB=whole body. Only OTU with relative abundance greater than 0.1% were included in the analysis.

8.4.4 Functional pathway analysis plots

The functional pathway analyses revealed an FDR significant pathway based on the hip T-score classification and 5 FDR significant pathways based on the FN classification (Fig. 8-7 to 8-12). The interferon gamma receptor 1 orthology group (IFNGR1/CD119) was common for the BMD sites and was significantly higher for the osteopenic/osteoporotic groups both for hip and FN classification than the healthy counterparts.

The interferon gamma receptor 1 orthology group (K05132) was significantly higher in abundance (FDR p-value < 0.039) for the osteopenic/osteoporotics (OP) compared to the healthy (H) groups. Similarly, based on the FN osteoporosis classification the OP was significantly (FDR p-value < 0.024) higher than the H group.

Figure 8-7 **The Interferon gamma receptor 1 (CD119) K05132 orthology group based on the hip osteoporosis classification.** Zero-inflated Gaussian fit, FDR p-value < 0.039

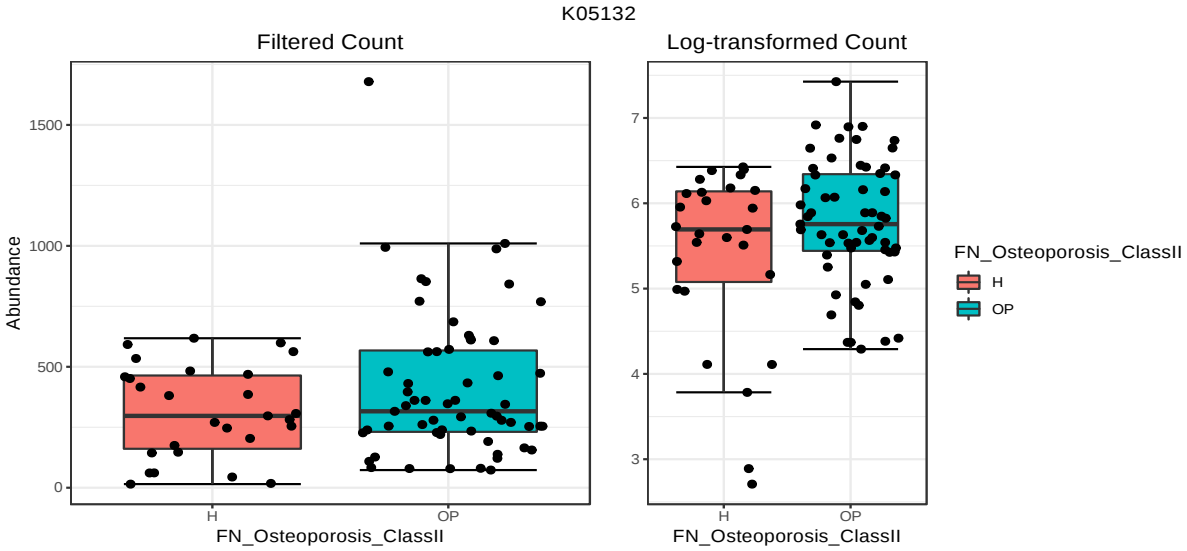

Figure 8-8 **The Interferon gamma receptor 1 (CD119) K05132 orthology group based on the FN osteoporosis classification.** Zero-inflated Gaussian fit, FDR p-value < 0.024

Conversely, the DNA-directed RNA polymerase II subunit (RPB11/POLR2J) K03008 orthology group was significantly (FDR p-value < 0.024) higher for the healthy (H) group than the osteopenic/osteoporotic women (OP).

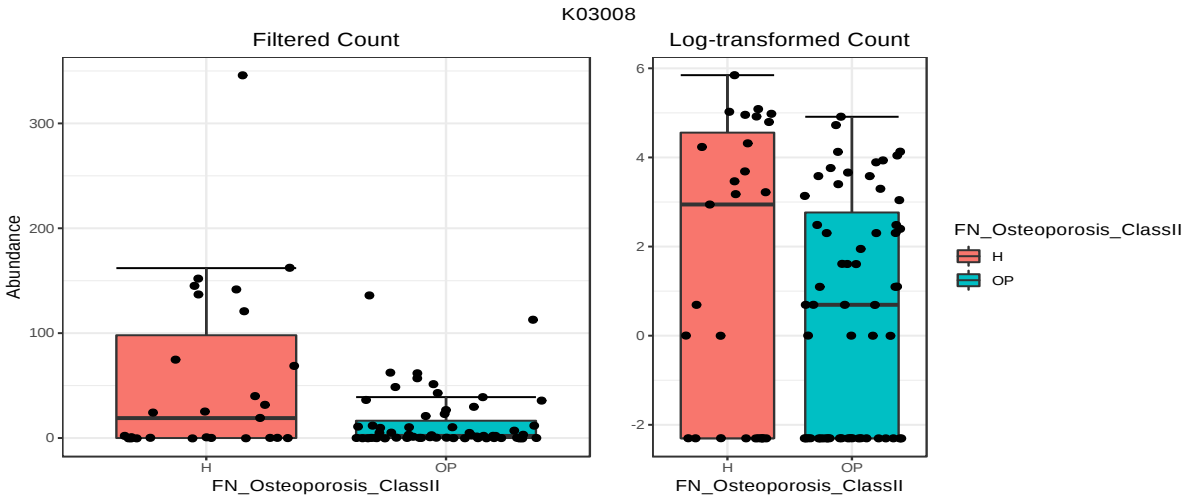

Figure 8-9 **The DNA-directed RNA polymerase II subunit (RPB11) K03008 orthology group based on the FN osteoporosis classification.** Zero-inflated Gaussian fit, FDR p-value < 0.024

The DNA-directed RNA polymerase subunit omega (rpoZ) K03060 orthology was also significantly (FDR p-value < 0.024) higher in abundance for the women with osteopenia/osteoporosis (OP) than the healthy (H).

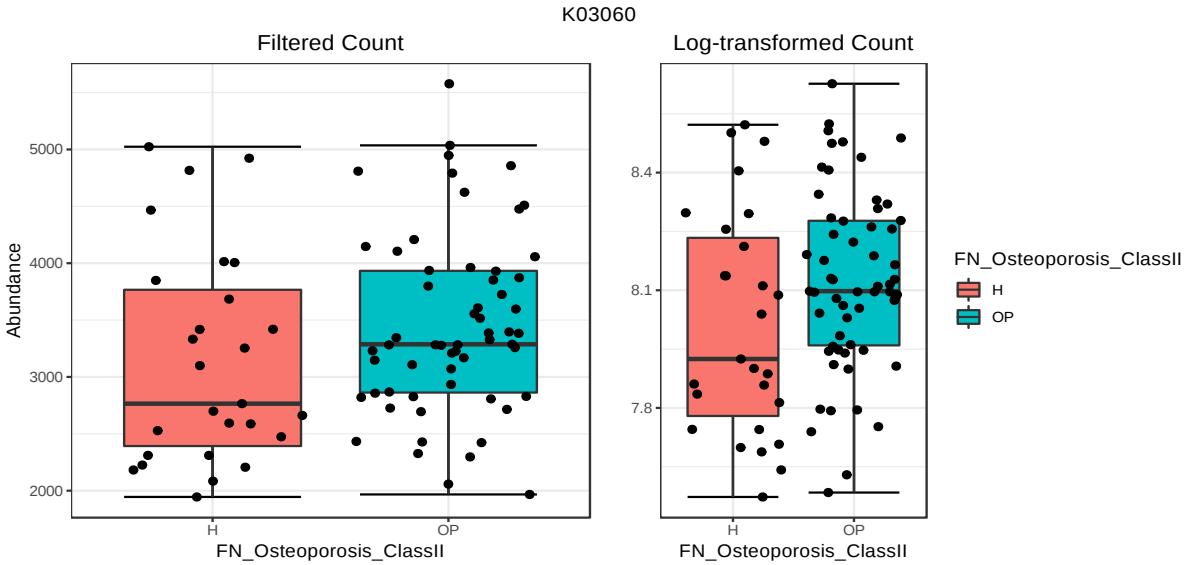

Figure 8-10 **The DNA-directed RNA polymerase subunit omega (rpoZ) K03060 orthology group based on the FN osteoporosis classification.** Zero-inflated Gaussian fit, FDR p-value < 0.024

The fructuronate reductase (uxuB) K00040 orthology group was significantly (FDR p-value < 0.039) higher amongst the osteopenic/osteoporotic than the healthy (H) groups.

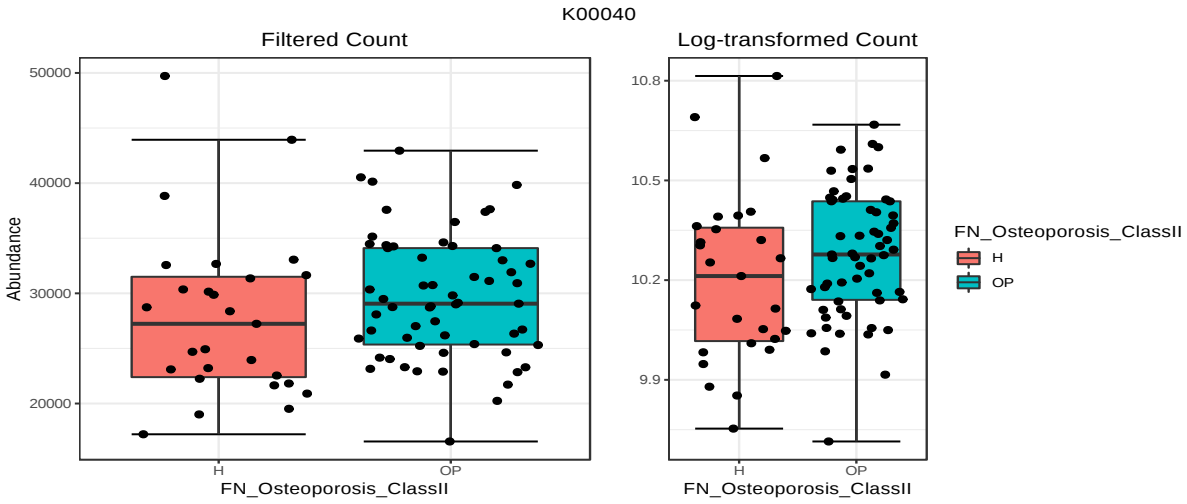

Figure 8-11 **The fructuronate reductase (uxuB) K00040 orthology group based on the FN osteoporosis classification.** Zero-inflated Gaussian fit, FDR p-value < 0.039

The nucleoside-diphosphate kinase (ndk/NME) K00940 orthology group was similarly significantly (FDR p-value < 0.024) higher among the osteopenic/osteoporotic (OP) groups than the healthy (H).

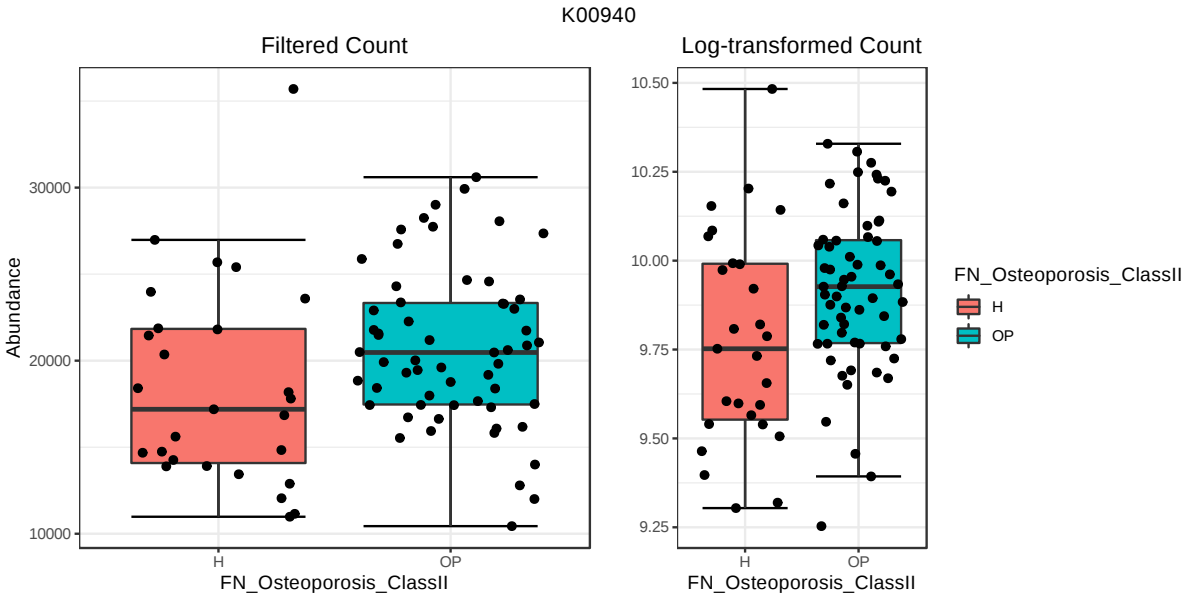

Figure 8-12 **The nucleoside-diphosphate kinase (ndk/NME) K00940 orthology group based on the FN osteoporosis classification.** Zero-inflated Gaussian fit, FDR p-value < 0.024

8.5 Discussion

Although two studies [14, 17] have researched the relationship between the microbiota and bone health, this study, to our knowledge is the first to use the whole metagenomics sequencing method for DNA sequencing to generate composition and predictive function of the microbiota.

8.5.1 Alpha and Beta diversity

In this study, utilising shotgun metagenomic sequencing by employing the HiSeq®2500 System, we found that alpha diversity of the microbial profiles differed based on the hip and FN classifications of osteoporosis status. Higher diversity was observed among the healthy group compared to the osteopenic/osteoporotic group. In addition, beta diversity using the PCoA by Bray-Curtis index indicated a difference for the analyses based on the FN classification but was not significant for classifications based on the hip and spine osteoporosis status.

8.5.2 Correlations

Correlations between relevant microbes and BMD values revealed positive correlations between *Lactobacillus, Bacillus, Paenibacillus* and BMD at all sites. Of note, is the positive correlation between *Alkaliphilus*, an alkaliphilic bacterium and BMD. Extreme alkaliphiles are sources of useful extracellular enzymes such as alkaline protease, alkaline cellulose and alkaline lipase [18]. A typical alkaphilic *Bacillus* are useful to uptake nutrients and rotate the flagellum [19]. This mild alkalinity is therefore important due to the effect of an acidic environment which could cause the loss of bone and stimulate osteoclastogenesis.

Meanwhile, *Bacteroides* and *Parabacteroides* were negatively correlated with BMD values based on all the BMD sites. In contrast to the findings of Li et al. (2019), *Roseburia* was negatively and poorly correlated with BMD at all sites. Similarly, *Bifidobacterium* was poorly correlated with BMD. However, similar results as Li et al. (2019) were found for Lactobacillus, which was positively correlated with BMD at all sites [17]. Rather surprisingly, *Lachnospiraceae*, although known as an important SCFA producer, was weakly correlated with BMD at all sites.

The *Firmicutes,* such as *Lactobacillus* and *Faecalibacterium* produce lactate and butyrate respectively. They are known for their role in mucin synthesis by the Goblet cells, a specialised epithelial cell of the epithelium, thus maintaining integrity which is important for gut health [20]. Several studies on mouse models [21, 22], a rat model [23-25] and a human clinical trial [26] have reported the effect of the probiotics containing *Lactobacillus* species on bone health. It is well-known that the *Lactobacillus* species can inhibit bone loss by decreasing osteoclastogenesis as is indicated by the results of this study showing a positive correlation between the genus *Lactobacillus* and BMD at all sites [2]. This finding however contradicted the findings by Das et al. 2019, which reported that *Lactobacillus* was more abundant in subjects with osteoporosis compared with the control groups [14]. This study's findings showing negative correlations between *Bacteroides* and BMD were similar to that of Li et al. 2019, however, they failed to show the correlations between the *Parabacteroides*, *Enterococcus* and bone density [17].

The correlations in this study were strong while others were weak. The strength of the correlations may be due to the BMD sites, calcium metabolism or the variabilty of the bone health measurement at a particular site.

8.5.3 Functional analysis and pathway

The interferon gamma receptor 1 (CD119) orthology group was found to be higher in the OP groups than the H group for both the hip and FN T-score classifications. This orthology group corresponds to several immune pathways: cytokine-cytokine receptor interaction, HIF-1 signaling, necroptosis, osteoclast differentiation, JAK-STAT signaling and Th1, Th2 and Th17 cell differentiation. The natural killer cell mediated cytotoxicity pathway is also linked to this orthology group.

HIF-1 signaling pathway has been linked to severity of osteoporosis after menopause [27]. Likewise, the JAK-STAT pathway which is a key intracellular mediator of metabolic cytokines through IL-6 family signals has been linked to osteoporosis [28]. Th1 and Th2 cell differentiation has both been linked with bone resorption [29]. Th17 cells produce IL-17 and IL-17 promotes bone loss [30]. All of these pathways have been reported to be catabolically linked to skeletal muscle degeneration and wasting, metabolic diseases (diabetes), cancer and ultimately osteoporosis [28, 30].

This result however contradicts that of Zhang et al (2017) which investigated healthy teenage male patients' periodontal cells *in vitro* and stated that these pathways were osteogenic [31]. The varying results may be as a result of the crosstalk of key pathways differences due to the age group, sample representation and methodology.

Interferon gamma receptor 1 binds interferon gamma (the sole member of the interferon type II) which in early history is known as the immune interferon [32]. Interferon gamma is produced by activated T cells while the gene IFNGR1 encodes IFN-γR1 which is found on macrophages. The receptor of IFN-γ initiates the biological effects of IFN-γ through its own receptor system. Earlier, about 60 years ago; IFN-γ was known for its antiviral properties but in the ensuing years its immune and inflammatory roles were discovered [33]. IFN-γ interacts with IFN-γ receptor exerting pleiotropic effects at the cell surface.

The DNA-directed RNA polymerase II subunit (RPB11) K03008 orthology group was higher amongst the H than the OP groups. The organism responsible for the function is *Saccharomyces cerevisiae* (strain ATCC 204508 / S288c) (otherwise known as Baker's yeast) and the molecular function include DNA binding, protein dimerization and RNA polymerase activity [34].
However, the DNA-directed RNA polymerase subunit omega (rpoZ) K03060 orthology group was higher amongst the OP women. The rpoZ is linked to *Escherichia coli* (strain K12), while one of the functions is to promote RNA polymerase assembly.
DNA-directed RNA polymerase complexes are proteins that catalyses RNA synthesis by adding ribonucleotide units to a RNA chain using a DNA template. These orthology groups are also linked to the RNA polymerase pathway (genetic information processing); this enzyme synthesises RNA from a DNA template for the transcription process. This transcription process has been linked to increase in osteoblasts and bone formation [35, 36]. This is in accordance with the result of this study with a higher abundance of RPB11 for the healthy (H) group. On the other hand, rpoZ which is also a transcription enzyme and function as antibiotic biosynthesis was higher for the osteopenic/osteoporotic. This may be as a result of the immune system acting as the host's defense system for the osteopenic/osteoporotic group.

Fructuronate reductase (uxuB) K00040 orthology group was higher with the osteopenic/osteoporotic groups. This orthology group is related to the pentose and glucuronate interconversions (a part of carbohydrate metabolism) as well as the metabolic pathways. The organism that is related to these pathways is the *Escherichia coli* (strain K12). The catalytic activity is responsible for converting NAD^+ to NADH.
The nucleoside-diphosphate kinase (ndk/NME) K00940 orthology group is linked to the following pathways; purine metabolism, pyrimidine metabolism, metabolic pathways and biosynthesis of secondary metabolites. The organism responsible for this function is also *E.coli* (strain K12). The main role is in the synthesis of nucleoside triphosphate other than ATP, it is also known for its catalytic activity and phosphorylation at SER-119 and SER-121 [37, 38].

The uxuB and ndk were also higher with the osteopenic/osteoporotic (OP) groups than their healthy counterparts. Metabolic pathways are composed of the catabolism and anabolism actions. It seems that the rate of catabolism is higher for the osteopenic/osteoporotic (OP). In this age group, depending on the level of physical activity the rate of the metabolism may differ from an active young adult as well. Also considering the age, which might be a factor [39].

This study is the first investigation to use shotgun metagenomic sequencing to analyse gut microbiota in relation to bone health in a larger cohort of postmenopausal women. Nevertheless, there were some limitations observed in the study such as the cross-sectional nature preventing any direct causation and lack of host-associated variables and factors (e.g. dietary patterns and inflammation) that may mediate the link between the gut microbiota and bone density. In addition, this study included a small group of women with osteoporosis therefore the women were categorised into two groups.

8.5.4 Conclusion

In conclusion, lower gut microbial composition diversity in women with bone disorders is in agreement with other studies where lower diversity has been observed in 'disease' states versus healthy controls. *Bacteroidetes Bacteroides* and *Firmicutes Enterococcus* were identified as important putative biomarkers that are associated with the bone status of both the hip and femoral neck. This is significant due to the fact that these are the most frequent sites where fractures occur. *Acidimicrobium*, *Akkermensia* and *Ethanoligenens* were higher in abundance amongst the H groups compared to the OP groups. The interferon gamma receptor 1 (IFNGR1, CD119) orthology group may play a greater role in bone resorption especially for postmenopausal women. These findings support the concept that gut microbiota influences bone metabolism in postmenopausal women. The present results provide a basis for a longitudinal intervention to assess the role of gut microbiota composition and function in prevention of osteoporosis.

8.6 References

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Chapter 9 Discussion, Conclusion and Areas of Further Research

Owing to the huge burden of osteoporosis and the occurrence of fragility fractures, there is the need for descriptive, comparative and experimental evidence-based investigations to prove or disprove previous studies on bone health maintenance in postmenopausal women. Of the ageing population, women are more at risk after menopause due to oestrogen deficiency. This need drove and led to the recruitment of 127 postmenopausal women from the Manawatu-Wanganui region of New Zealand. With the New Zealand health system actively promoting nutrition literacy and physical education, the focus and future direction still remains active prevention of accidents and debilitating diseases such as falls, fractures and osteoporosis.

Although physical activity has been established to be beneficial for all ages, previous studies evaluating the role of lean mass on bone health status have generated inconsistent results especially for postmenopausal women [1, 2]. In this study, self-reported physical activity was positively associated with heel ultrasound parameters and bone density measures. This is in accordance with expectations and other theories that have referred to physical activity as the most important non-pharmacological approach to prevent fractures in people at risk of osteoporosis [3]. Of note was the positive association between lean mass and the heel ultrasound parameters as well as bone health measures.

The first question in this study sought to determine the relationship between body composition and bone health status in the cohort of postmenopausal women. Obesity (high BMI) was positively associated with increased bone density which was related to the ‘obesity paradox’ [4]. However, lean mass was observed as the most significant determinant of bone health status for this age group which is similar to the findings from other studies [5, 6].

The next question aimed to answer what types of nutrients and nutrient patterns (generated from 3-DDD) contributed by association to bone health status in postmenopausal women. Focussing on bone areas critical to osteoporosis, such as the spine, femoral neck and hip BMD; calcium, protein, phosphorus, vitamin B2 (riboflavin) and vitamin B3 (niacin) were all positively associated with BMD for the post-menopausal women. The result of this study indicated a positive relationship between B-vitamins and BMD. It was also found that nutrient patterns with high factor loadings of riboflavin, phosphorus and calcium was positively related to BMD at all sites. To date, little attention has been paid to the relationship between these B-vitamins and BMD in postmenopausal women and this suggests a future area of research.

The third question was posed to investigate the relationship between dietary patterns (obtained from FFQ) and bone health status in postmenopausal women. The result indicated that a dietary pattern with a high factor loadings of milk and milk-rich beverages was positively associated with spine T-score and whole-body BMD. The significance of milk intake and its role as a source of calcium, protein, phosphorus and B-vitamins is a well-researched area of study. As an important source of calcium, milk intake also increases the bioavailability of this important nutrient for bone metabolism. The milk and milk-rich beverages dietary pattern included a high factor loading of dairy foods and coffee. Intake of coffee from a recent study of 2,507 menopausal women has also been linked to improvement in mood, memory and concentration [7]. This result is in accordance with other studies as is evident from the referenced reviewed work of Movassagh and Vantaparast (2017) [8] and Denova-Gutierrez et al. (2018) [9]. Park et al. (2012) also recruited 1,725 postmenopausal women in Korea reaching a conclusion that dairy and green tea dietary patterns may reduce risks of osteoporosis [10]. A dietary pattern with a high factor loading of oily fish, sports drink and seafood was also negatively associated with total body fat percentage. This dietary pattern is similar to the Mediterranean diet pattern which has been linked to a lower body composition and weight loss [11].

The fourth question was regarding the relationships between inflammatory status, BMD and bone biomarkers. Based on the results of this study, the findings showed that 13 inflammatory markers measured by the electrochemiluminescence immunoassay analyser (ECLIA) were higher among women with osteoporosis while 2 inflammatory markers (CRP and TNF-α) were lower among this group. The results indicated both pro-inflammatory and anti-inflammatory cytokine levels were higher amongst the osteoporotic group which could be due to the synergistic role of cytokines in maintaining immune balance in inflammatory response [12].

The correlations showed a negative association observed for IL-1β and spine BMD/T-score as well as IL-12p70 and hip and spine BMD/T-score. This result may indicate that an increase in IL-1β and IL-12p70 circulation in the blood is related to lower BMD and T-scores in post-menopausal women. CRP and IFN-α2 were also negatively correlated with bone biomarkers (P1NP and CTX-1), which may suggest an increase in the circulatory CRP and IFN-α2 is related to the bone remodelling activity. Correlations between $25(OH)D_3$ and inflammatory markers showed positive correlations except for CRP and TNF-α, possibly indicating the evidence for vitamin $25(OH)D_3$ as an anti-inflammatory factor of the innate immune system. Reports have shown body fat parameters such as BMI, fat percentage and fat mass to be positively associated with low-grade inflammation [13, 14].

These associations were illustrated further in this study, showing positive correlations between all fat measures and markers of inflammation such as CRP and IL-6. Generally high body fat percentage, an indicator of obesity was associated with increased inflammation and lower levels of anti-inflammatory cytokines. Furthermore, a positive correlation was observed between ferritin and spine T-score. Although not significant, positive correlations were observed between ferritin and all BMD sites and T-scores.

The last question in this research study inquired about the association between the gut microbiome and bone health status based on WHO classification of osteoporosis amongst post-menopausal women. The relationship between the gut composition and predictive function of women and their bone density classified into healthy and osteopenic/osteoporotic groups was investigated.

The findings of this study showed that alpha diversity of the microbial profiles differed based on the hip and femoral neck osteoporosis classifications. Meanwhile, beta diversity principal component analysis by Bray-Curtis index showed differences based on femoral neck classifications only.

Positive correlations were observed between *Lactobacillus, Bacillus, Paenibacillus* and *Geobacillus* (all from the phylum Firmicutes) and BMD at all sites. However, negative correlations were reported for *Bacteroides, Parabacteroides* and all BMD sites. This result was in accordance with previous studies that have reported the importance of the *Lactobacillus* species in bone maintenance [15, 16] and was similar to the work by Li et al. (2019) showing a negative correlation between *Bacteroides* and BMD [17].

Furthermore, the interferon gamma receptor 1 (CD119) orthology group linked to cytokine-cytokine receptor interaction, HIF-1 signaling, necroptosis, osteoclast differentiation, JAK-STAT signaling and Th1, Th2 and Th17 cell differentiation was found to be higher in abundance in relation to both the hip and femoral neck BMD classifications. The natural killer cell mediated cytotoxicity pathway is also linked to this orthology group. These pathways have been reported to be catabolically linked to skeletal muscle degeneration and wasting, metabolic diseases (diabetes), cancer and ultimately osteoporosis [18, 19].

The present study reiterates the importance of physical activity and LM in postmenopausal women. In addition, over time chronic inflammation as shown by high levels of inflammatory markers could be detrimental to bone density. This chronic inflammation determined mainly by diet and hormones could also be linked to the gut microbiota status of an individual.

9.1 Conclusion

The main goal of the present study was to investigate the relationship between diet, inflammation, gut microbiota and bone health in postmenopausal women. This thesis has provided a deeper insight to the risk factors that co-exists for osteoporosis and these findings will be of interest to the dairy industries, the scientific community and women in general.

In conclusion, the findings reported in this thesis have pointed out the significance of physical activity, lean body mass, milk and milk-rich dietary pattern, inflammation status and gut microbiota for bone maintenance in postmenopausal women. Gut microbiota modulation is an emerging concept for enhancing all areas of human health. To date, no study has reported the microbial functional differences between healthy and osteopenic/osteoporotic postmenopausal women.

The scope of this study was limited in terms of the sample size and the cross-sectional nature limiting generalisability. Despite its exploratory nature, this study offers some insight into the co-existing risk factors for osteoporosis in older aged women.

9.2 Areas of future research

Based on the relationship between the dietary patterns and bone results, research into the bioactive components of coffee that might be beneficial for bone health is necessary.

Further human intervention study is also needed to provide a quantifiable effect of probiotic, prebiotic and synbiotic products on bone density.

Preliminary evidence from this study suggests that microbial IFNGR1 (CD119) is an important orthology group for bone health in postmenopausal women. Further metagenomics and metabolomics techniques needs to be employed to determine actual rather than predicted functional capacity differences in gut microbiome between healthy and osteopenic/osteoporotic postmenopausal women.

The effect of the gut microbiome on the modulation of diseases should not be considered in isolation because of several factors known to influence its composition and function, e.g. the host genetics e.g. geography/environment, age, diet, sleep pattern etc.

Compositional taxonomic approaches used in studies are limited. Metagenomic analyses and data are needed to inform on gene abundance and predict which genes are being expressed (microbiome metabolomics) in relation to differences between the disease state and the healthy.

Research focus has been on rodents however, the murine microbiome is different from human microbiome [20]. Similarly, murine cells are different from human cells both in mitochondrial density and metabolic rate as well as in fatty acid composition of their phospholipids membrane. Murine cell membranes are known to contain a higher polyunsaturated fatty acid docosahexaenoic acid [21]. Also, there are differences in body mass/size, life history, diet variety and rate of aging. Therefore, larger cross-sectional study that could lead to focussed human intervention studies modulating the gut microbiome to benefit or aid prevention of osteoporosis is warranted.

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Appendices

Appendix 1 Mean selected nutrient intakes across quartiles of nutrient pattern scores (For Chapter 5)

Consumption patterns of selected nutrients across quartiles of nutrient patterns give a description of the nutrient patterns with Q1 being the lowest quartile and Q4 the highest quartile (Table A1)

Table A1: Mean (SD) of selected nutrient intakes across quartiles of nutrient pattern scores (n=101)

	NP1					NP2				
Nutrients	**Q1**	**Q2**	**Q3**	**Q4**	***P*-value**	**Q1**	**Q2**	**Q3**	**Q4**	***P*-value**
Protein (g)	68.9 (18.0)	81.4 (16.7)	94.0 (13.6)	97.8 (24.3)	<0.001	83.9 (21.8)	82.4 (24.6)	89.7 (22.6)	86.5 (16.7)	0.651
Total fat (g)	70.3 (16.3)	77.7 (30.1)	87.1 (16.0)	82.6 (22.4)	0.049	71.3 (20.2)	71.3 (22.3)	85.8 (22.3)	89.8 (19.6)	0.002
Carbohydrate (g)	136.0 (35.3)	166.2 (47.7)	203.5 (40.5)	262.1 (53.1)	<0.001	195.1 (61.5)	190.8 (68.1)	194.9 (71.6)	187.4 (59.2)	0.971
Vitamin D (µg)	7.1 (9.2)	5.4 (4.6)	6.9 (7.0)	5.7 (3.8)	0.703	3.3 (2.1)	5.3 (3.9)	5.2 (3.6)	11.4 (10.1)	<0.001
Calcium (mg)	652.5 (193.5)	809.1 (184.6)	940.1 (248.2)	1315.0 (388.5)	<0.001	985.0 (404.1)	871.5 (321.7)	945.2 (404.8)	917.8 (307.3)	0.723
Magnesium (mg)	290.9 (54.5)	322.2 (67.3)	411.7 (76.7)	462.2 (80.2)	<0.001	321.5 (84.4)	335.5 (93.8)	389.1 (82.7)	444.2 (81.8)	<0.001
Phosphorus (mg)	1177.0 (224.2)	1345.4 (236.8)	1624.0 (180.2)	1931.1 (359.7)	<0.001	1504.0 (472.7)	1420.3 (382.1)	1569.0 (370.1)	1592.2 (283.2)	0.381
Potassium (mg)	2787.1 (456.2)	3216.8 (479.5)	3896.6 (770.7)	4542.8 (842.4)	<0.001	3359.0 (849.8)	3424.5 (859.8)	3748.4 (901.4)	3930.2 (1045.9)	0.095
Total omega 3	1.5 (0.9)	1.5 (1.2)	1.4 (0.9)	1.5 (1.2)	0.989	0.8 (0.4)	1.0 (0.5)	1.7 (1.3)	2.2 (1.1)	<0.001
Total omega 6	5.9 (2.6)	7.9 (6.4)	7.5 (3.5)	6.9 (2.9)	0.340	4.8 (1.9)	5.5 (2.2)	7.6 (3.0)	10.3 (5.9)	<0.001
Beta-carotene (µg)	2214.0 (1659.4)	1954.0 (1705.2)	2970.5 (1532.3)	3057.2 (2366.4)	0.089	1529.0 (783.7)	2107.6 (1388.8)	2520.0 (1649.7)	4073.6 (2364.8)	<0.001
Alpha-carotene (µg)	294.9 (336.0)	312.9 (661.1)	640.0 (506.7)	692.5 (716.8)	0.021	230.2 (259.8)	408.2 (435.6)	409.7 (584.0)	901.5 (777.1)	<0.001
Biotin (µg)	8.2 (5.8)	5.6 (3.4)	13.1 (13.3)	10.8 (15.1)	0.073	6.4 (5.6)	8.8 (9.5)	11.2 (14.4)	11.5 (11.8)	0.302
Vitamin C (mg)	82.1 (32.5)	125.9 (80.2)	135.3 (70.1)	128.7 (74.8)	0.024	104.1 (55.6)	102.4 (48.2)	141.6 (97.6)	125.2 (62.6)	0.139
Vitamin B12	3.5 (2.2)	3.5 (1.3)	3.6 (1.1)	6.4 (6.7)	0.012	3.5 (1.5)	3.9 (1.8)	4.9 (4.4)	4.7 (5.8)	0.490
Saturated fat	25.6 (8.1)	29.9 (13.5)	31.3 (7.6)	35.2 (15.0)	0.035	32.7 (13.6)	28.4 (11.1)	32.6 (12.8)	28.5 (9.4)	0.368
Cholesterol (mg)	296.2 (157.6)	299.4 (165.2)	313.3 (129.3)	270.3 (135.7)	0.771	266.6 (95.7)	292.8 (160.0)	355.3 (163.5)	265.2 (144.4)	0.098
Dietary fibre (g)	21.1 (4.5)	22.6 (5.3)	29.5 (8.4)	35.4 (8.9)	<0.001	23.8 (8.7)	23.7 (6.0)	27.5 (7.4)	33.9 (9.8)	<0.001
Energy kJ	6529.2 (813.1)	7548.5 (1626.7)	8818.1 (945.5)	9515.0 (1632.7)	<0.001	7797.6 (1733.7)	7791.4 (1973.9)	8414.5 (1713.8)	8448.4 (1416.0)	0.333
MET-minutes	310.2 (359.4)	252.1 (216.9)	292.6 (245.9)	800.5 (1433.2)	0.036	438.8 (699.5)	533.9 (1233.6)	333.7 (553.1)	339.4 (307.0)	0.771

One-way ANOVA was used to test the difference across the quartiles.

Appendix 2 Mean selected nutrient intakes across quartiles of nutrient pattern scores continued (For Chapter 5)

Table S1: Mean (SD) of selected nutrient intakes across quartiles of nutrient pattern scores (n=101) contd.

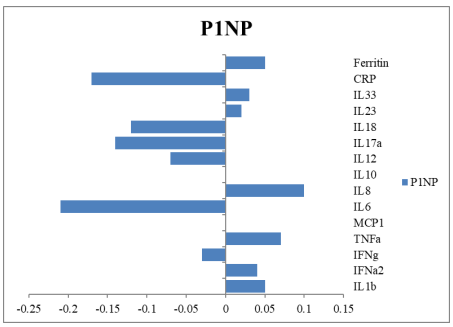
			NP3		
Nutrients	**Q1**	**Q2**	**Q3**	**Q4**	***P*-value**
Protein (g)	69.2 (16.5)	84.5 (18.4)	89.9 (18.3)	98.9 (21.9)	<0.001
Total fat (g)	59.5 (17.3)	71.4 (13.8)	82.0 (15.8)	105.3 (13.0)	<0.001
Carbohydrate (g)	182.4 (62.3)	194.9 (64.8)	179.8 (58.2)	210.9 (70.8)	0.305
Vitamin D (µg)	4.8 (6.2)	6.2 (4.5)	8.7 (9.6)	5.4 (3.7)	0.163
Calcium (mg)	887.4 (419.8)	971.3(318.4)	886.4 (321.9)	970.4 (379.2)	0.716
Magnesium (mg)	343.8 (100.5)	376.5 (107.7)	355.7 (76.1)	412.7 (94.0)	0.064
Phosphorus (mg)	1333.2 (410.8)	1532.9 (382.0)	1526.4 (294.1)	1688.6 (371.8)	0.011
Potassium (mg)	3570.5 (1031.1)	3692.8 (964.3)	3555.3 (826.0)	3632.8 (949.4)	0.952
Total omega 3	1.3 (1.2)	1.2 (0.6)	1.6 (1.0)	1.6 (1.3)	0.452
Total omega 6	6.6 (6.4)	6.6 (3.0)	6.8 (2.4)	8.2 (3.8)	0.441
Beta-carotene (µg)	3512.5 (2584.7)	2614.4 (1959.0)	2405.6 (1244.5)	1677.4 (801.7)	0.005
Alpha-carotene (µg)	779.1 (864.1)	524.8 (563.9)	407.1 (393.7)	233.8 (271.9)	0.009
Biotin (µg)	5.2 (3.2)	7.0 (4.7)	8.2 (6.4)	17.5 (17.9)	<0.001
Vitamin C (mg)	151.4 (88.3)	115.7 (71.9)	103.4 (49.6)	102.3 (52.6)	0.039
Vitamin B12	4.8 (6.9)	3.9 (1.7)	3.6 (1.4)	4.7 (2.5)	0.613
Saturated fat	20.4 (5.7)	27.9 (8.3)	32.4 (10.1)	41.5 (11.6)	<0.001
Cholesterol (mg)	194.6 (93.1)	245.4 (102.9)	353.7 (133.2)	388.1 (160.5)	<0.001
Dietary fibre (g)	28.9 (9.9)	27.7 (10.7)	25.1 (7.9)	26.9 (7.1)	0.509
Energy kJ	6973.7 (1425.0)	7833.4 (1565.3)	8125.4 (1363.3)	9517.7 (1581.1)	<0.001
MET-minutes	608.0 (1341.6)	365.1 (629.7)	326.4 (340.3)	353.0 (347.8)	0.549

One-way ANOVA was used to test the difference across the quartiles.

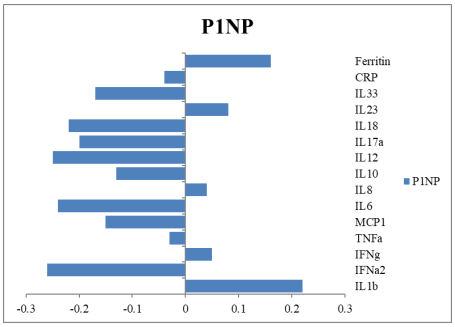
Appendix 3 Comparison correlation coefficients of grouped bar graphs of bone biomarkers with inflammation markers (For Chapter 7)

Fig. 1: P1NP

Healthy



Osteopenic



Osteoporotic

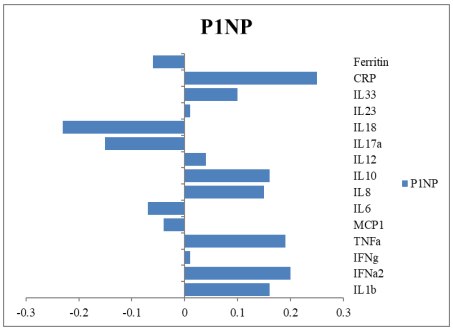
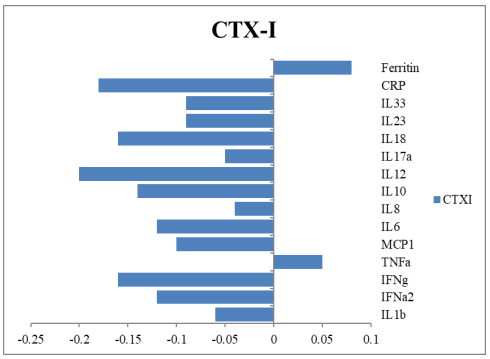


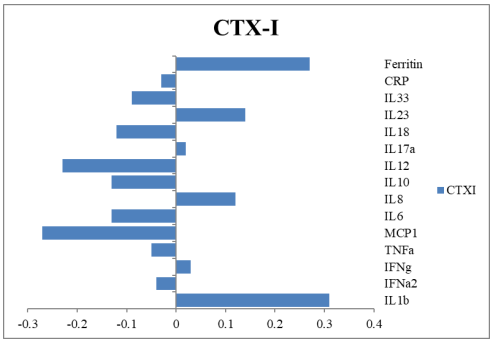
Figure 1 shows the correlation coefficients of the inflammation markers against P1NP in groups according to their osteoporotic status. P1NP was mainly negatively correlated with inflammation markers for healthy and osteopenic and mainly positively correlated for osteoporotic group.

Fig. 2: CTX-1

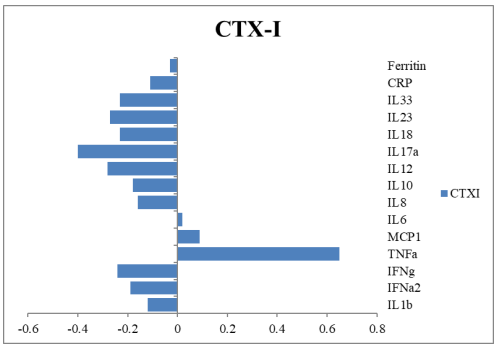
Healthy



Osteopenic



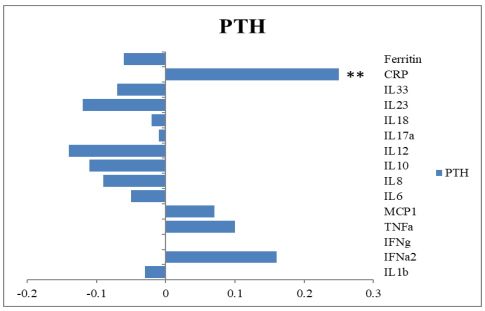
Osteoporotic



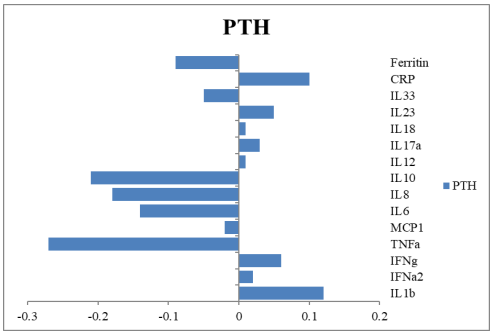
As shown in Fig. 2, TNF-α is predominantly increased with the bone resorption marker CTX-1. Similarly, IL-6 and MCP-1 are positively correlated with CTX-1 for the osteoporotic group. Whereas for the healthy, ferritin and TNF-α and for the osteopenic group, ferritin, IL-23, IL-18, IL-8, IFN-γ and IL-1β was positively correlated.

Fig. 3: PTH

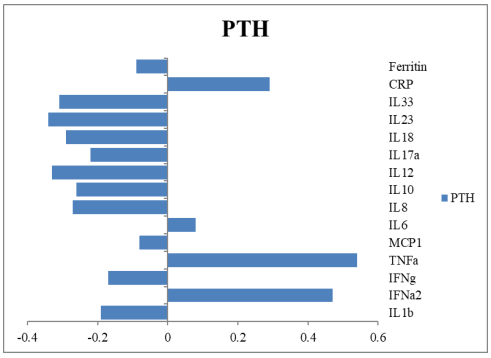
Healthy



Osteopenic



Osteoporotic

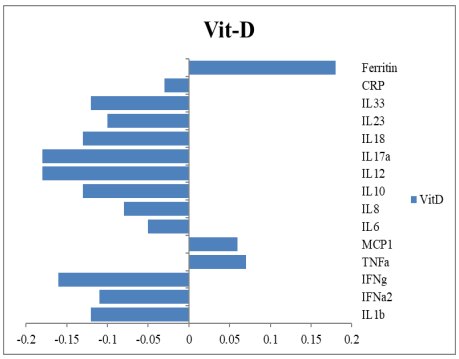


* p < .05, **p < .01, *** p < .001

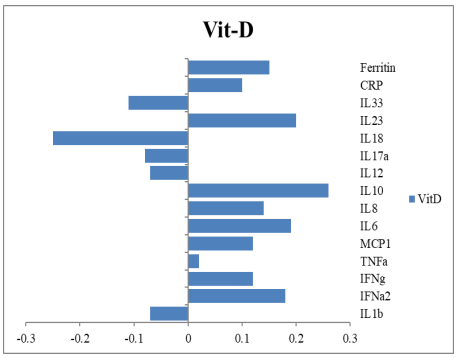
Figure 3 shows the relationships between PTH and the cytokines. As can be seen in the osteoporotic group, the synthesis of PTH stimulates the production of CRP, IL-6, TNF-α and IFN-α2. This is in accordance with other research that have investigated the relationship between PTH and cytokines [1, 2].

Fig. 4: Vit. D

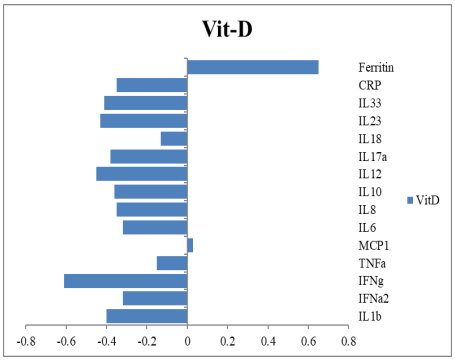
Healthy



Osteopenic



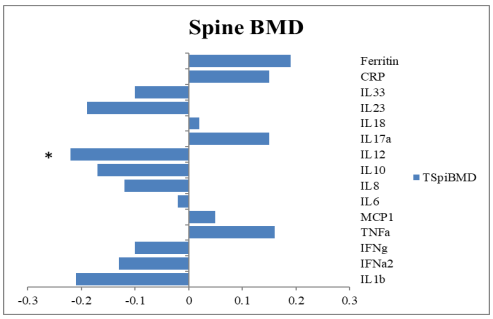
Osteoporotic



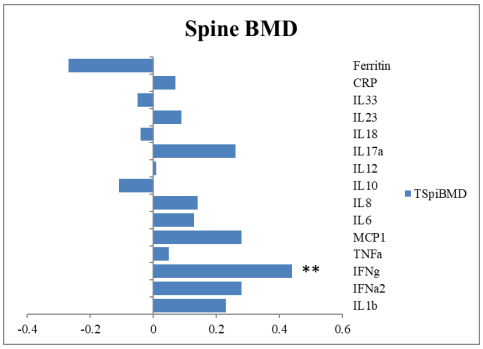
As can be visualized in Fig. 4, in the osteoporotic group there are negative correlations of vitamin D and the cytokines and CRP except for ferritin. This again is in accordance with the literature on immune cell studies, confirming the anti-inflammatory effect of vitamin D [3].

Fig. 5: Spine BMD

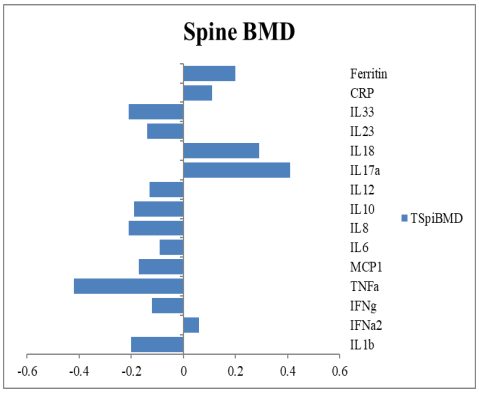
Healthy



Osteopenic



Osteoporotic



* p < .05, **p < .01, *** p < .001

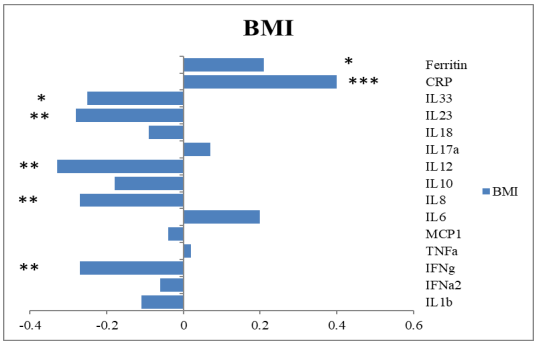
Figure 5 shows that in the osteoporotic group, most of the inflammatory markers are negatively correlated except for ferritin CRP, IL-18, Il-17A and IFNα2. The osteopenic group in comparison was mainly positively correlated with spine BMD.

Appendix 4 The relationship between inflammatory markers and obesity across groups (For Chapter 7)

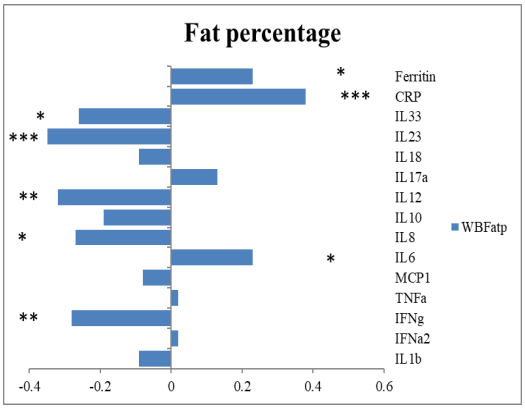
The bar graphs show the relationship between markers of obesity and the 15 inflammation markers. Levels of CRP were the most strongly correlated with BMI, whole body fat percentage and fat mass (WBFatp and WBFat).

Fig. 6: Inflammation markers and BMI, Fat percentage and Fat mass

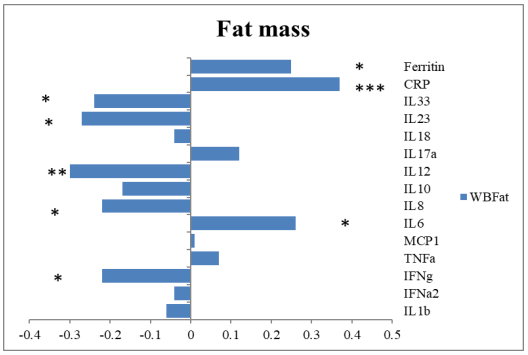
BMI



Fat percentage



Fat mass



* p < .05, **p < .01, *** p < .001

From the graph, we observed positive correlations between ferritin, CRP, IL-17A, IL-6 and BMI, fat percentage and fat mass. All other cytokines were negatively correlated with BMI, fat percentage and fat mass.

Appendix 5 Matrix Plot of Weight whole body fat versus IL-12, IL-33 groups (For Chapter 7)

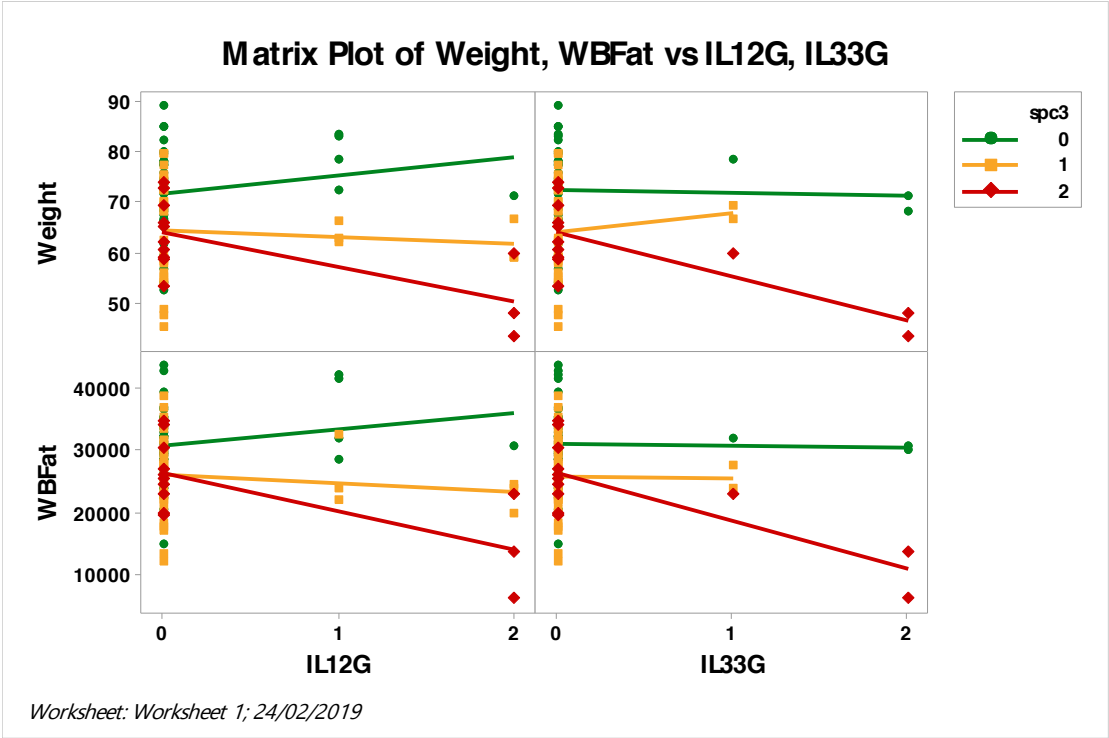


Fig. 7: Matrix Plot of Weight whole body fat versus IL-12, IL-33 groups

Key

Spc3 (spine osteoporotic classification into three groups)	IL12G and IL33G
0 – Healthy	0 - <LOD (level of detection)
1 – Osteopenic	1 – Low Conc (< median)
2 – Osteoporotic	2 – High Conc (> median)

As shown in Fig. 7, IL-12 increases as weight increased in the healthy spine group and decreases as weight decreased in the osteoporotic group and the trend similar to whole body fat mass. Trend is however, slightly dissimilar for the healthy groups of the IL-33.

Appendix 6 Rarefactions

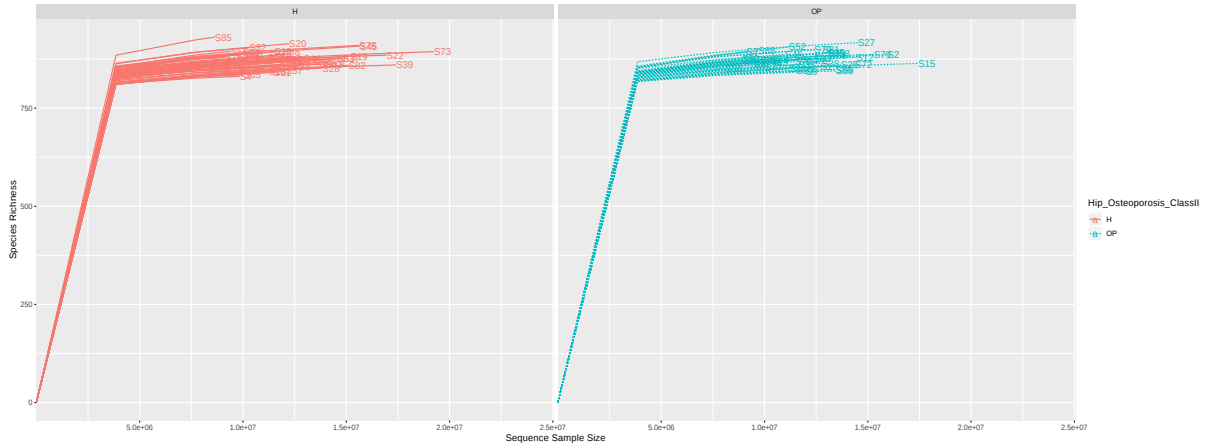


Fig. 1: Rarefaction based on the hip osteoporosis classification

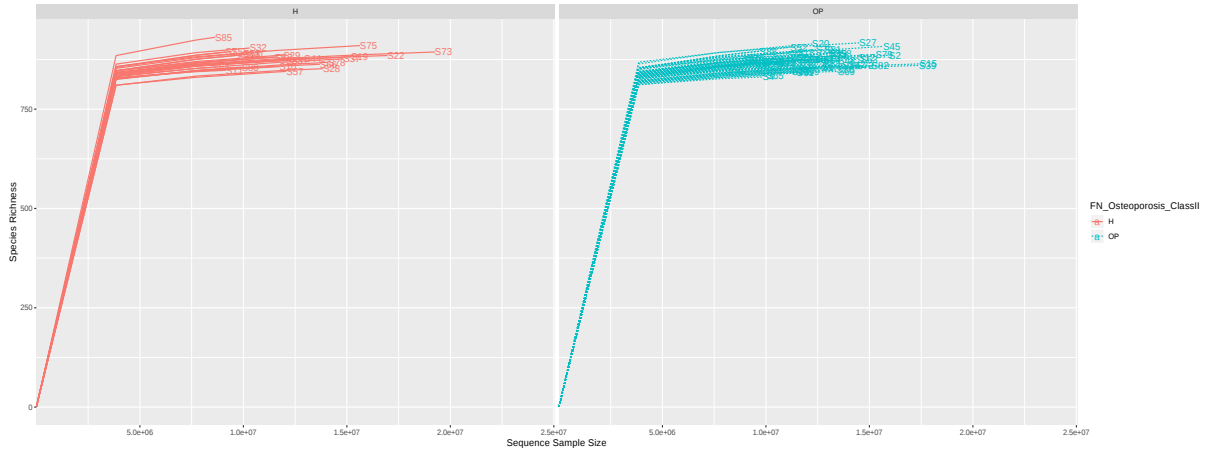


Fig. 2: Rarefaction based on the femoral neck osteoporosis classification

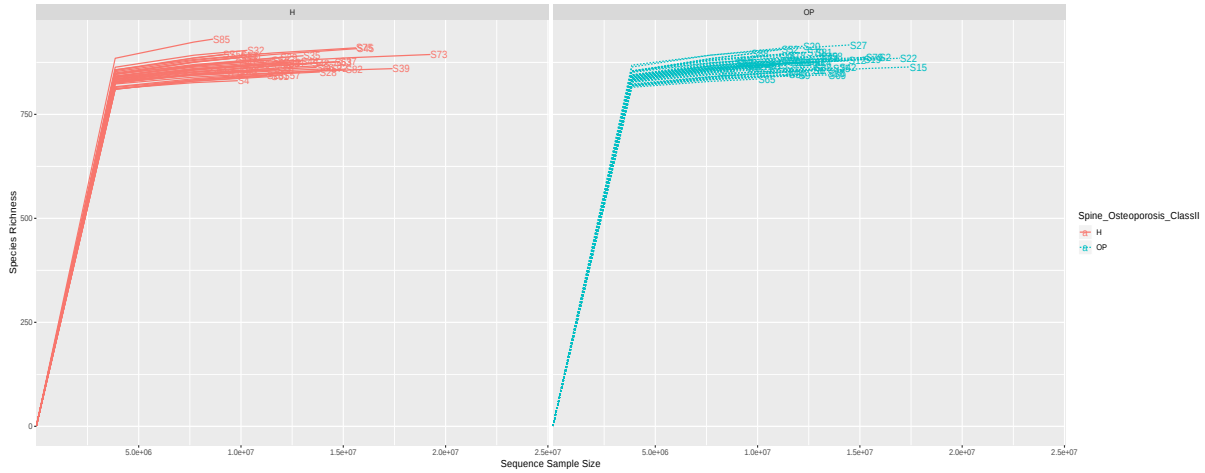


Fig. 3: Rarefaction based on the spine osteoporosis classification

Appendix 7 ANOSIM, ADONIS AND PERMDISP (For Chapter 8)

1. ANOSIM

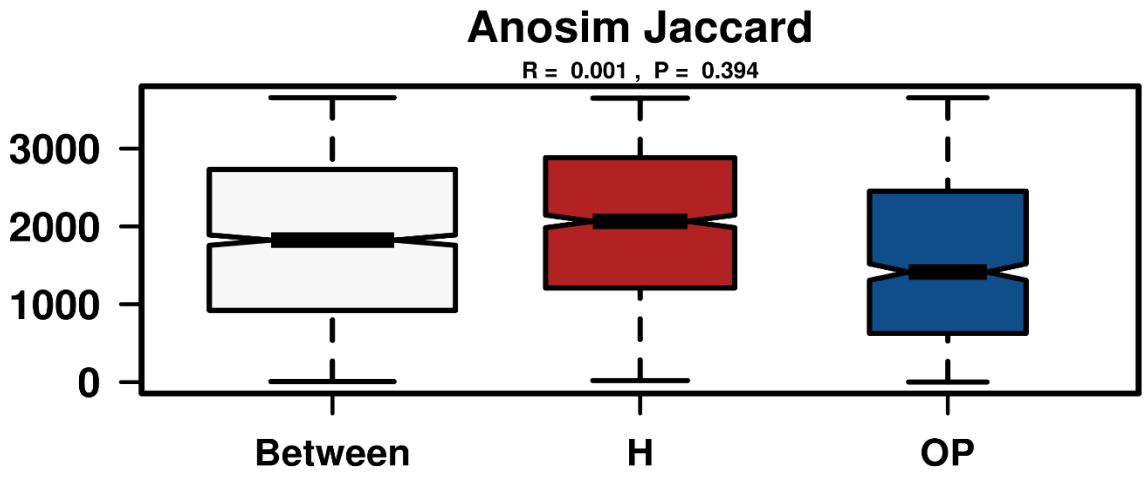


Fig. 1: ANOSIM based on the hip osteoporosis classification using Jaccard index

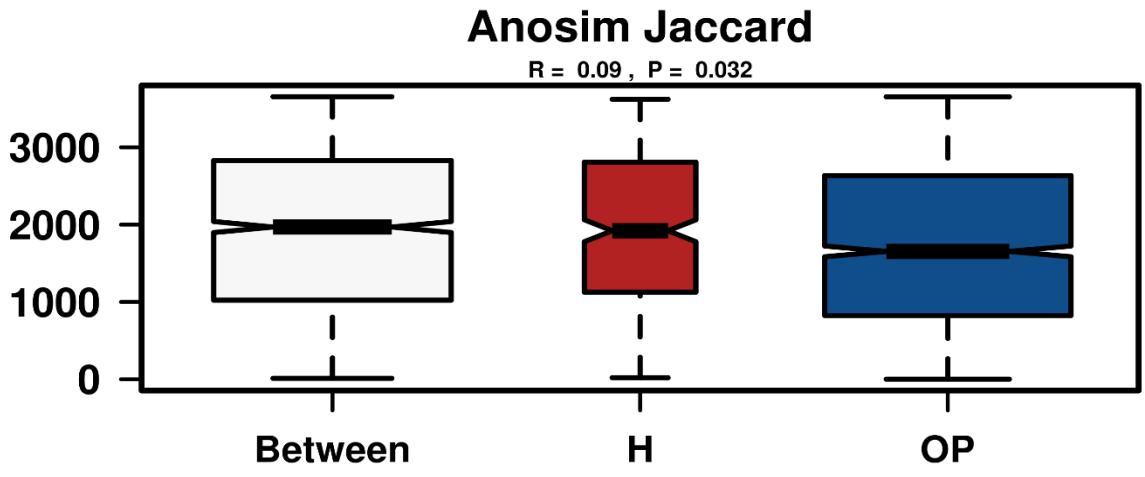


Fig. 2: ANOSIM based on the FN osteoporosis classification using Jaccard index

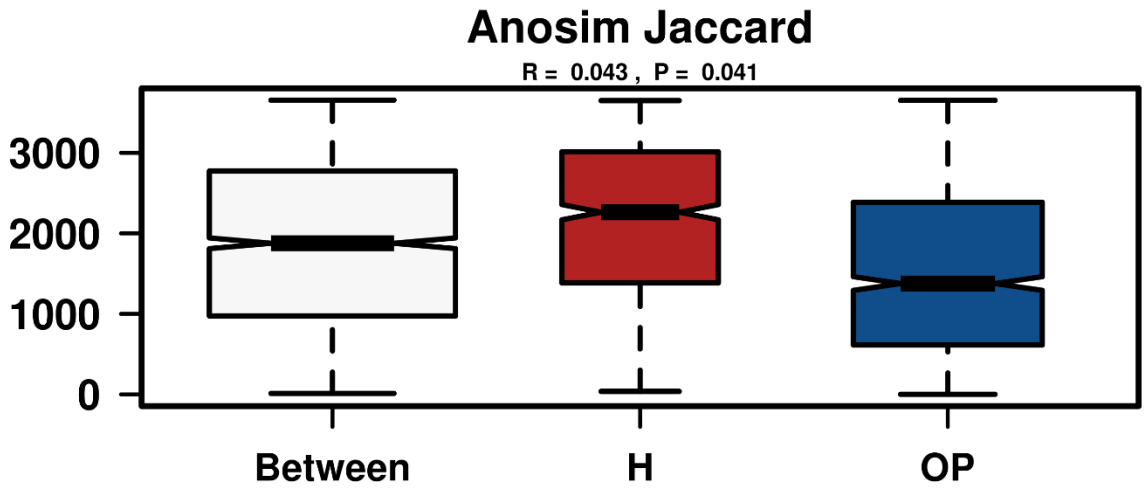


Fig. 3: ANOSIM based on the spine osteoporosis classification using Jaccard index

2. ADONIS

Adonis OTU Jaccard
Model P-value:

	R2	P
Hip_Osteoporosis_ClassII	0.0214	0.066

Adonis OTU Jaccard
Model P-value:

	R2	P
FN_Osteoporosis_ClassII	0.0348	0.00666

Adonis OTU Jaccard
Model P-value:

	R2	P
Spine_Osteoporosis_ClassII	0.0112	0.44

3. PERMDISP

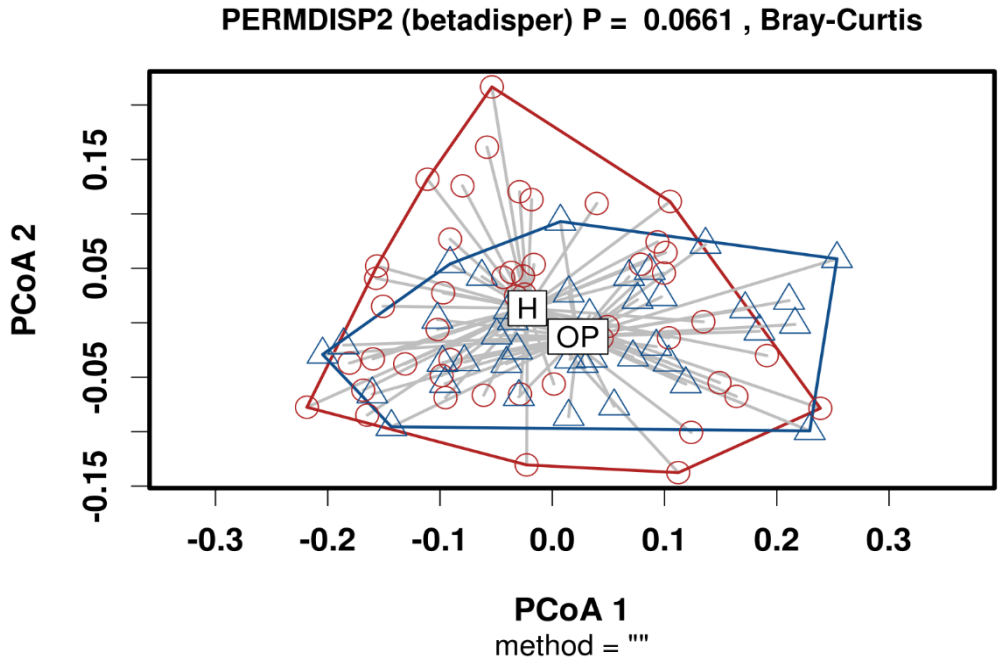


Fig. 4: Permdisp according to hip osteoporosis status using the Bray-Curtis index

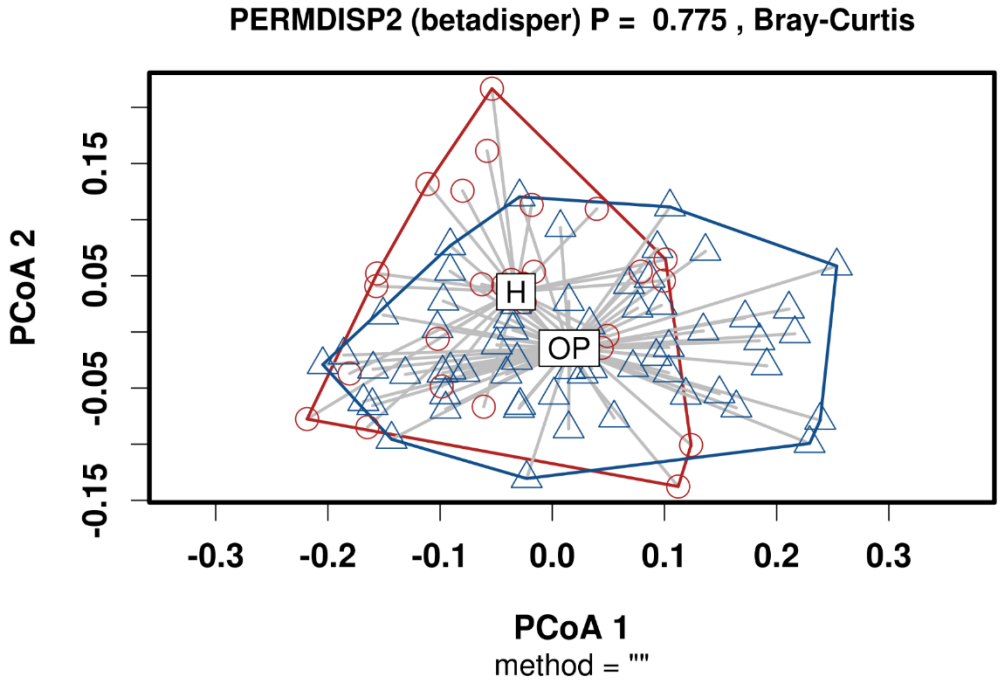


Fig. 5: Permdisp according to FN osteoporosis status using the Bray-Curtis index

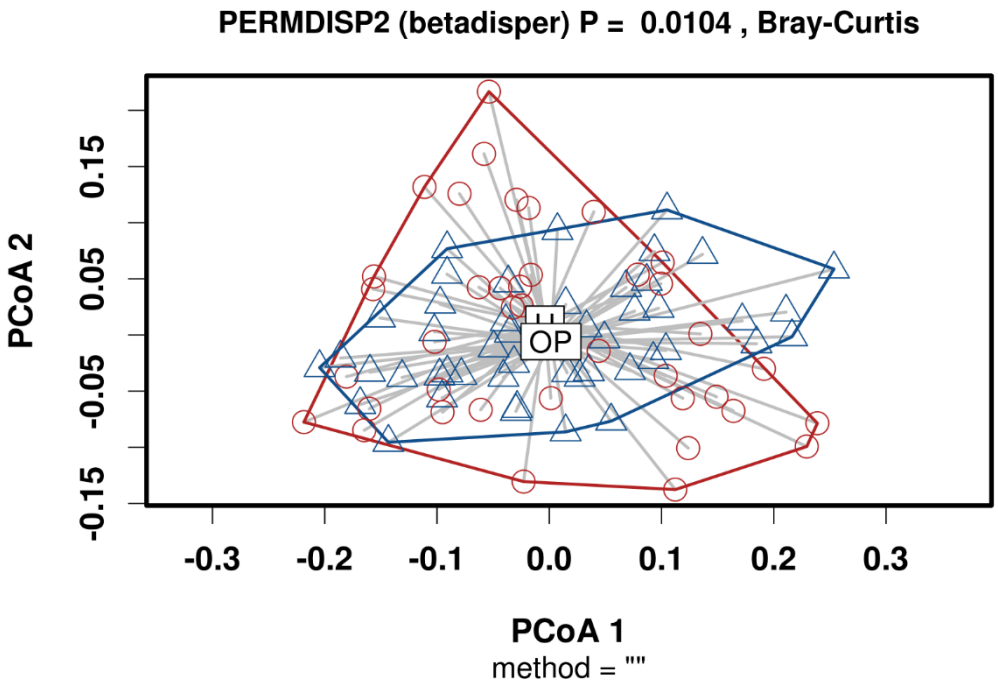


Fig. 6: Permdisp according to spine osteoporosis status using the Bray-Curtis index

Appendix 8 Functional analysis plots

The microbial gene functional analysis PCA plots of the microbial gene function according to the osteoporosis status showed an overlap and did not show a clear separation between the healthy and osteopenic/osteoporotic women (Fig. 8.10 – 8.12). The figures show that there was 10.9% variation on the y-axis and 38.7% on the x-axis.

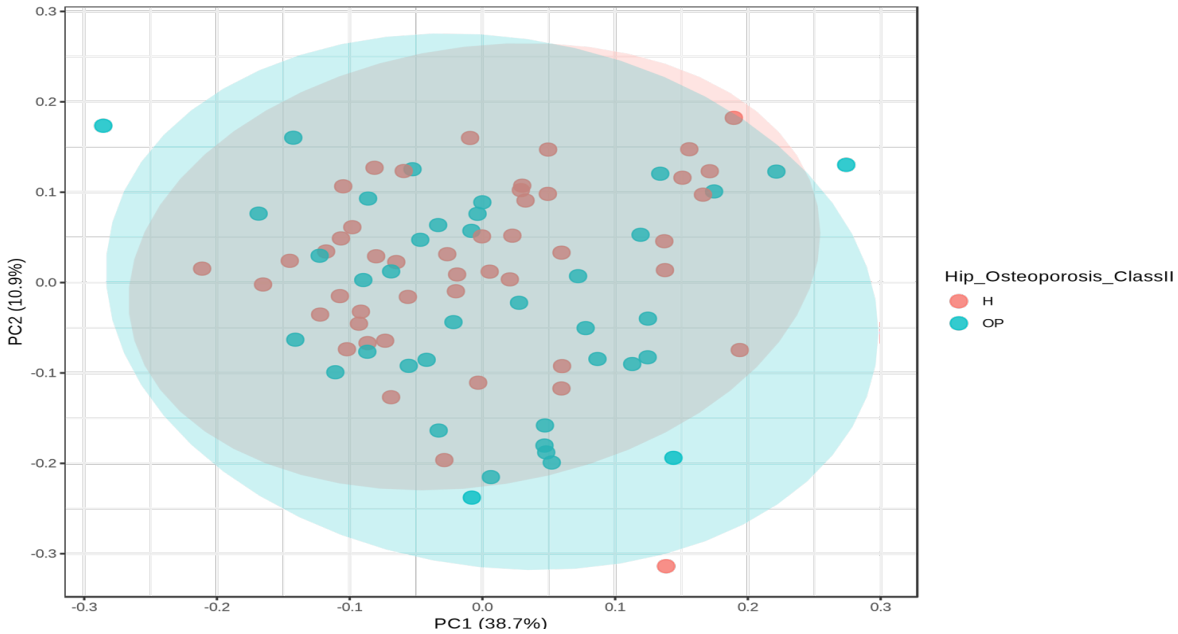


Fig. 8.10: Functional analysis plot for the hip osteoporosis classification

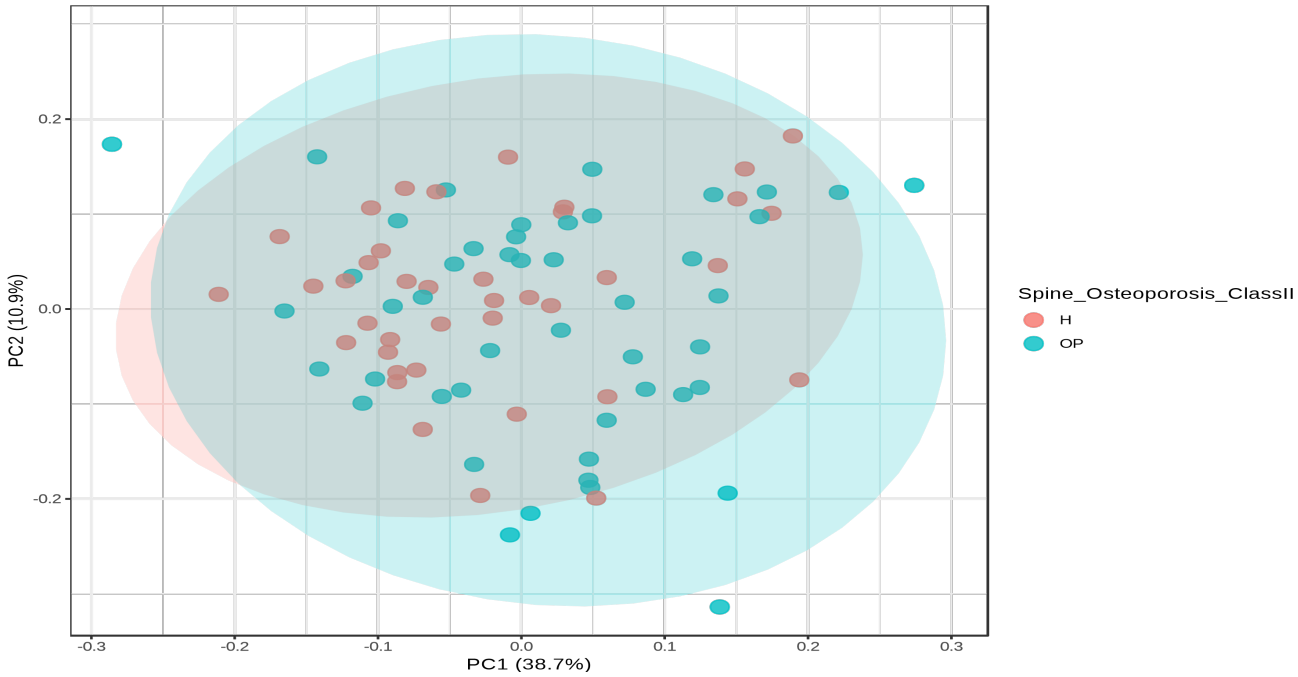


Fig. 8.12: Functional analysis plot for the spine osteoporosis classification

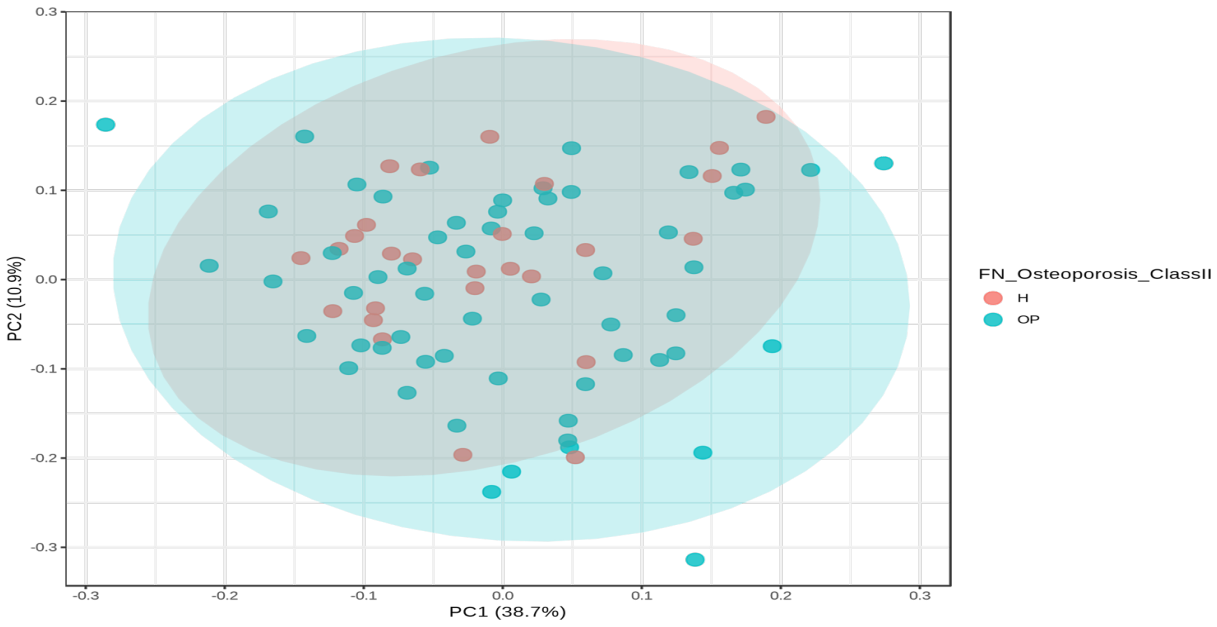


Fig. 8.11: Functional analysis plot for the FN osteoporosis classification

Appendix 9 Posters

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Cytokine Production, Ferritin Levels and Bone Health in Postmenopausal women

ACKNOWLEDGEMENTS
Supporting Technical Staff
Palmerston North Medical Research Foundation
Allen Foundation, USA
Fonterra Cooperative Group Ltd.

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Background

Osteoporosis is a major public health concern which, as a result of the demineralisation of bones, leads to increased fracture risks[1]. Studies have indicated that low-grade inflammation, due to the effect of pro-inflammatory cytokines such as interleukin (IL)-1β and IL-6 impairs DNA repair and leads to cellular and immunological senescence as well as biological ageing[2]. Ferritin is an acute phase reactant which also serves as a marker of inflammation.

In postmenopausal women, low-grade inflammation, coupled with reduced oestrogen levels, lead to an increase in bone resorption which partly counters increase in bone formation[3] resulting in increased bone turnover.

Materials and Methods

Eighty-six postmenopausal women aged between 54 and 81 years participated in the "Bug'n'Bones", a cross-sectional study. Body composition and bone mineral density were measured using the Dual energy X-ray Absorptiometry (DXA). Cytokine assays were prepared according to the BioLegend® LEGENDplex™ Multi-Analyte Flow Assay kit instructions and measured using a Beckman Coulter's Gallios flow cytometer. Plasma ferritin levels were measured using an electro-chemiluminescence immunoassay (ECLIA).

Massey University Human Ethics Committee approved this study: Southern A, Application 17/17.

Objectives

1. To examine the relationship between levels of inflammatory markers and bone health status (classified by the World Health Organization classification of osteoporosis) in apparently healthy postmenopausal women.
2. To evaluate differences in markers of inflammation such as IL-10, an anti-inflammatory cytokine, pro-inflammatory cytokines and chemokine levels amongst the normal, osteopenia and osteoporotic groups.

Results

Figure 1 provides an overview of the mean levels of plasma inflammatory markers in the whole group of women. As illustrated, monocyte chemoattractant protein (MCP-1) was observed at the highest levels amongst all the cytokines measured.

Meanwhile the results of analysis of variance among the groups according to the osteoporotic classification are shown in Fig.2. The plasma levels of IFN-α2 (*P*=0.027), IFN-γ (*P*=0.009), IL-12p70 (*P*=0.049), IL-33 (*P*=0.048) and MCP-1 (*P*=0.05) were significantly higher in the osteoporotic group compared to the osteopenic or normal groups. However, rather surprisingly, plasma CRP levels (*P*=0.044) was significantly lower amongst the osteoporotic than osteopenic and normal groups.

In addition, Fig.3 and Fig.4 showed that IL-10 and IL-33 levels were significantly higher in women with low ferritin levels (*P*=0.001 and *P*=0.002 respectively).

Fig.1: Mean Plasma Levels of Inflammatory Markers

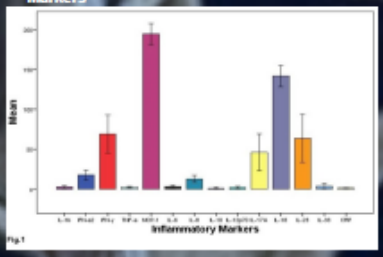


Fig.3: Mean Plasma Ferritin Levels by IL-10 status

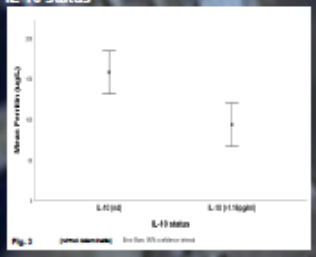


Fig.2: Plasma Levels of Inflammatory Markers by Osteoporosis Classification

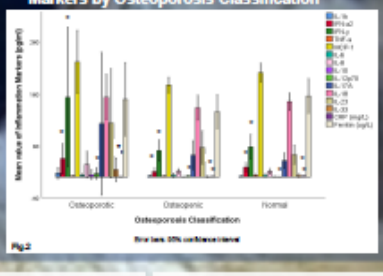
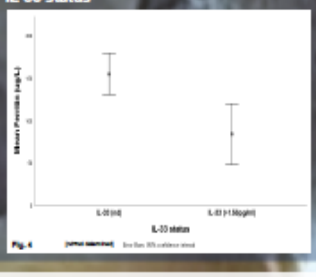


Fig.4: Mean Plasma Ferritin Levels by IL-33 status



Discussion

The course of inflammageing status is multi-factorial, resulting from not only immunosenescence but also important factors such as dietary pattern, obesity and the gut microbiota status[3]. Studies have indicated the contribution of IL-17 and IL-23 but not interferon-γ (IFN-γ) to osteoporosis. Likewise, IL-10 but not IL-33 has been linked to lower ferritin levels and low iron stores[4].

Conclusions

High plasma levels of IFN-α2, IFN-γ, IL-12p70, IL-33 and MCP-1 in apparently healthy postmenopausal women are associated with low bone mass. In addition, an increase in levels of IL-10 may be associated with low ferritin status in women of this age group. This results need to be investigated further due to the complex interplay of comorbidities and cytokines that could potentially affect acute phase reactants such as CRP and ferritin.

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2. Fuggle et al. Osteoporosis International. 2018:1-9.
3. Hsu & Pacifici. Calcified tissue International. 2017:1-10.
4. Tilg et al. Journal of Immunology. 2002; 169(4):2204-9.

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The association between gut microbiota and bone health status

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Background

The gut microbiota, otherwise known as the largest gene pool of the human body may play a significant role in bone health maintenance (Zhang et al. 2018). The gut microbiota have been postulated to affect nutrient absorption, endocrine and immune systems involved in bone metabolism.

The aim was to determine the relationship between gut microbiota and bone health status in postmenopausal women. We sought to identify if there were any differences in diversity between healthy (H) and osteopenic or osteoporotic (OP) women.

Materials & Methods

This cross-sectional study examined 127 postmenopausal women aged between 54 - 81 years. Body composition and bone mineral density (BMD) parameters were determined using dual-energy X-ray absorptiometry (DXA). The faecal DNA samples underwent library preparation by Nextera XT DNA library prep kit (Illumina, USA) and shotgun metagenomic sequencing was conducted on an Illumina HiSeq® 2500 System. Shotgun metagenome data was processed in MG-RAST and analysed and were visualised in Calypso.

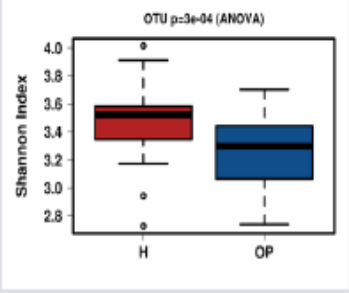


Fig.1. Alpha diversity defined by femoral neck T-score classification

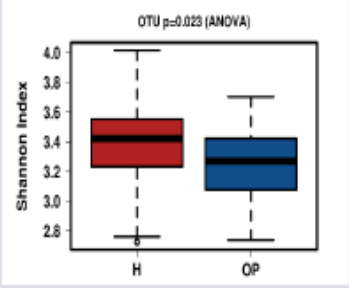
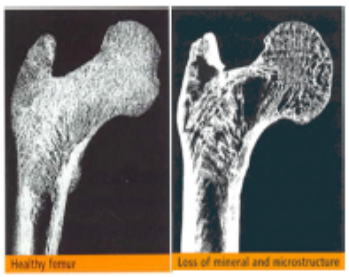


Fig. 2. Alpha diversity defined by hip T-score classification



Results

Microbial composition diversity (alpha-diversity by Shannon index) was significantly decreased in the OP group of women with low BMD of the hip (P=0.023) and femoral neck (P=0.0003) compared to the healthy group. There were no significant differences based on the spine BMD classification (P=0.48) .

Based on the classification for all the BMD sites, *Bacteroidetes bacteroides* were significantly higher in abundance for the OP compared to the healthy (H) groups.

Linear discriminant analysis effect size (LEfSe) identified bacterial OTUs ($p < 0.05$) associated with hip bone status but none for spine status. These included *Bacteroides* and *Parabacteroides* which were increased in OP women while *Bacillus* and *Lactobacillus* were increased in the healthy groups.

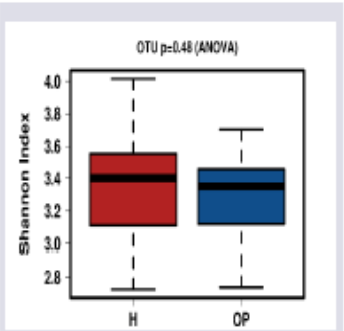


Fig. 3. Alpha diversity defined by spine T-score classification

References
1. Zhang et al. Intractable & rare diseases research. 2018: 7(3), 148 –155
2. Mu et al. Frontiers in Immunology. 2017: 8 (590)
3. Brown et al. PLoS One. 2011: 6(10):e26792

Discussion

The phylum Bacteriodetes is commonly found in most Westerners. *Bacteroides* are symbiotics which assist digest foods and produce important nutrients for energy. *Bacteroidetes* such as *Bacteroides* converts lactic acid into other short chain fatty acids (SCFA) for example acetic acid and formic acid. Although *Bacteroides* are useful in protecting against pathogenic bacteria, if present in too large quantities, they may damage the lining of the gut, causing inflammation and autoimmune diseases (Mu et al. 2017).

However, the *Firmicutes*, such as *Lactobacillus* and *Faecalibacterium* produce lactate and butyrate respectively. They are known for their role in maintaining a healthy gut via mucin synthesis on the intestinal epithelial layer for gut integrity (Brown et al. 2011).

Conclusions

Lower gut microbial composition diversity in women with bone disorders is in agreement with other studies where lower diversity has been observed in 'disease' states versus healthy controls. Our results provide a basis for a longitudinal intervention assessment of the role of gut microbiota composition and function in osteoporosis to potentially yield preventive and therapeutic interventions.

Fig. 4A

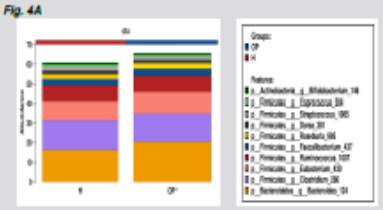


Fig. 4B

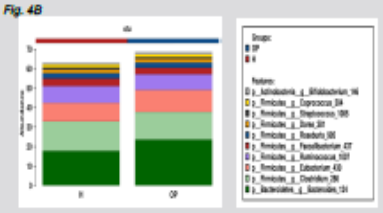


Fig. 4C

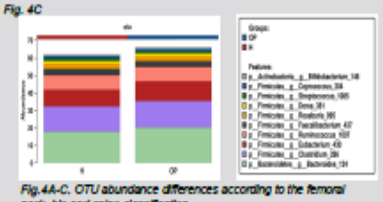


Fig.4A-C. OTU abundance differences according to the femoral neck, hip and spine classification

Dietary Patterns in relation to Body Composition and Bone Health in New Zealand postmenopausal women

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Background

Nutrition affects bone health. Dietary patterns (DP) generate insights into which particular combination of foods consumption patterns are beneficial and which may be limiting for bone and nutritional status (Denova-Gutiérrez et al. 2018). Most informed food choices, especially during senescence are paramount to health.

The aim of this study was to explore the associations between dietary patterns, bone and body composition in postmenopausal women.

Materials & Methods

This cross-sectional study examined 127 postmenopausal women aged between 54 - 81 years. Body composition and bone health status were determined using dual-energy X-ray absorptiometry (DXA). The dietary composition was assessed by a validated food frequency questionnaire (FFQ) composed of 108 foods, from which 34 food groups were created. Dietary patterns were identified by principal component analysis. The bone and nutritional parameters were regressed onto the dietary pattern.

Objective

We investigated the relationship between identified dietary patterns and bone mineral density (BMD) as well as body composition in New Zealand postmenopausal women.

Results

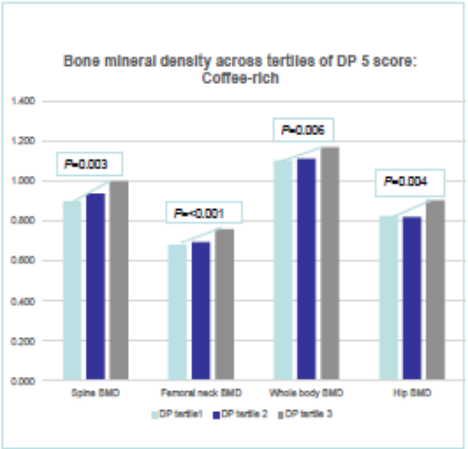
Five DPs namely, vegetable-rich, milk-based, fruit and carbohydrate, tea-rich and coffee-rich DPs were identified. Two of which were associated with body composition. The fruit and carbohydrate DP was associated with a reduction in weight ($r = -0.192$, $P = 0.038$), body mass index ($r = -0.184$, $P = 0.047$) and body fat ($r = -0.181$, $P = 0.050$). Coffee-rich DP was associated with increased weight ($r = 0.223$, $P = 0.015$), body mass index ($r = 0.245$, $P = 0.008$) and body fat ($r = 0.195$, $P = 0.036$). Bone health status; spine T-score ($r = 0.283$, $P = 0.002$), hip T-score ($r = 0.309$, $P = 0.001$), femoral neck T-score ($r = 0.317$, $P = 0.001$) and whole-body BMD ($r = 0.261$, $P = 0.004$) were also associated with coffee-rich DP. Milk-based DP was associated with increased spine T-score ($r = 0.304$, $P = 0.001$).

Discussion

A dietary pattern characterised by high factor loadings of coffee was associated with higher BMD and T-scores. Intake of fruit and carbohydrate DP was negatively correlated with weight, BMI and fat percentage. Even though some research work has reported coffee consumption (≥ 4 cups/day intake) as detrimental to bone health, a few others have reported the beneficial effect of moderate coffee intake. Of note is Choi et al., (2016) which reported protective benefits of coffee on bone among Korean postmenopausal women. The finding indicated the anti-inflammatory effects of kahweol, the oestrogenic effect of trigonelline and the antioxidant effect of non-flavonoid polyphenol in coffee. Additional, randomised controlled trials are therefore needed in this area to determine the beneficial effects of coffee intake.

Conclusion

Although coffee intake has been associated with low bone mass, the possibility of the impact or effects of coffee as an important determinant of bone health status in postmenopausal women needs more research focus.



Multiple linear regression of the dietary pattern scores by nutritional status

Parameters	Variance Explained (%)	β-coefficient	95% Confidence interval	P-value
Weight (kg)	10.1			
Vegetable-rich DP		0.001	-2.084, 2.104	0.992
Milk-based DP		0.094	-0.989, 3.199	0.298
Fruit and carbohydrate DP		-0.192	-4.353, -0.165	**0.036**
Tea-rich DP		0.069	-1.279, 2.909	0.442
Coffee-rich DP		0.223	0.528, 4.716	**0.016**
BMI	11.0			
Vegetable-rich DP		-0.006	-0.797, 0.746	0.948
Milk-based DP		0.121	-0.247, 1.296	0.181
Fruit and carbohydrate DP		-0.184	-1.571, -0.027	**0.049**
Tea-rich DP		0.045	-0.578, 0.965	0.620
Coffee-rich DP		0.245	0.292, 1.836	**0.007**
Total fat %	8.4			
Vegetable-rich DP		0.039	-1228.2, 1908.6	0.668
Milk-based DP		0.107	-639.6, 2497.2	0.243
Fruit and carbohydrate DP		-0.181	-3149.3, -12.6	**0.048**
Tea-rich DP		0.023	-1368.4, 1768.4	0.801
Coffee-rich DP		0.195	126.8, 3263.6	**0.034**

References

1. Denova-Gutiérrez et al. Nutrients 2018 Dec; 10(12): 1922.
2. Choi et al. PLoS One 2016 Jan; 11 (1): e0147762.

Appendix 10 Authors' contributions

Peer–reviewed papers	Author	Contribution
Lean body mass in the prediction of bone mineral density in postmenopausal women (Chapter 3)	Bolaji L. Ilesanmi-Oyelere	The candidate was involved with the research design, wrote the protocol for ethics approval, organised and collected the data, conducted the statistical analyses, wrote the first manuscript and read and reviewed the manuscript.
	Jane Coad	Prof. Coad was involved with the research design, collection of data. She read and reviewed the manuscript.
	Nicole C. Roy	Prof. Roy was involved with the research design, collection of data. She read and reviewed the manuscript.
	Marlena C. Kruger	Prof. Kruger had the idea, sourced the funding, was involved with the research design, data collection, read and reviewed the manuscript. Prof. Kruger is the candidate's main supervisor.
Associations between self-reported physical activity, heel ultrasound parameters and bone health measures in post-menopausal women (Chapter 4)	Bolaji L. Ilesanmi-Oyelere	The candidate was involved with the research design, wrote the protocol for ethics approval, organised and collected the data, conducted the statistical analyses, wrote the first manuscript and read and reviewed the manuscript.
	Nicole C. Roy	Prof. Roy was involved with the research design, collection of data. She read and reviewed the manuscript.
	Jane Coad	Prof. Coad was involved with the research design, collection of data. She read and reviewed the manuscript.
	Marlena C. Kruger	Prof. Kruger had the idea, sourced the funding, was involved with the research design, data collection, read and reviewed the manuscript. Prof. Kruger is the candidate's main supervisor.
The relationship between nutrient patterns and bone mineral density in postmenopausal women (Chapter 5)	Bolaji L. Ilesanmi-Oyelere	The candidate was involved with the research design, wrote the protocol for ethics approval, organised and collected the data, conducted the statistical analyses, wrote the first manuscript and read and reviewed the manuscript.

	Louise Brough	Dr. Brough was involved with the interpretations of the research.
	Jane Coad	Prof. Coad was involved with the research design, collection of data. She read and reviewed the manuscript.
	Nicole C. Roy	Prof. Roy was involved with the research design, collection of data. She read and reviewed the manuscript.
	Marlena C. Kruger	Prof. Kruger had the idea, sourced the funding, was involved with the research design, data collection, read and reviewed the manuscript. Prof. Kruger is the candidate's main supervisor.
Inflammatory markers and bone health in postmenopausal women: a cross-sectional overview (Chapter 8)	Bolaji L. Ilesanmi-Oyelere	The candidate was involved with the research design, wrote the protocol for ethics approval, organised and collected the data, was involved with the biochemical analyses, conducted the statistical analyses, wrote the first manuscript and read and reviewed the manuscript.
	Linda Schollum	Dr. Schollum provided interpretations of the analyses and revised the discussion section.
	Barbara Kuhn-Sherlock	Dr. Kuhn-Sherlock was involved with the statistical analyses.
	Michelle McConnell	Prof. McConnell was involved with the biochemical analyses.
	Sonya Mros	Mrs. Mros was involved with the biochemical analyses.
	Jane Coad	Prof. Coad was involved with the research design, collection of data. She read and reviewed the manuscript.
	Nicole C. Roy	Prof. Roy was involved with the research design, collection of data. She read and reviewed the manuscript.
	Marlena C. Kruger	Prof. Kruger had the idea, sourced the funding, was involved with the research design, data collection, read and reviewed the manuscript. Prof. Kruger is the candidate's main supervisor.

Appendix 11 Advertisement and Questionnaires

The relationship between dietary patterns, bone biomarkers, gut microbiome and bone density

Researchers from Massey University, Palmerston North, invites you to take part in a study to look at the relationship between the type of foods you eat, the bacteria in your gut and your bone strength. We are now investigating this in post-menopausal women, aged 55 to 70 years of age.

Phase 1

During the study period, after signing a consent form and reading the information sheet, we will ask you to fill-in a participants' questionnaire, 3-day diet diary, food questionnaire, an activity questionnaire, a bone and heel scan to assess the status of your bones.

Phase 2 (you will be invited after phase 1)

During this phase, urine, blood and faecal samples will be collected from participants by experienced and qualified professionals. Hygienic sample collection

We are looking for

- ✓ Healthy women aged 55 – 70 years
- ✓ Non smokers
- ✓ Five years past menopause
- ✓ Normal weight
- ✓ No food intolerance

You will receive voucher(s) for your contribution in each phase of this study.

If you are interested in taking part in this study, please contact Lilian Ilesanmi-Oyelere via e-mail on **bugsnbones2017@gmail.com or** [redacted]

Ethical approval

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 17/17. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 356 9099 x 85094, email humanethicsoutha@massey.ac.nz .

BUGS'n'BONES Study: The relationship between dietary patterns, bone biomarkers, gut microbiome and bone density

Researchers' introduction.

My name is Lilian Ilesanmi-Oyelere and I am undertaking this research as part of the requirements for my PhD in Nutritional Sciences. My supervisors are Professor Marlena Kruger and Professor Jane Coad in the School of Food and Nutrition at Massey University, Palmerston North and Dr. Nicole Roy based at AgResearch Grasslands in Palmerston North. This study is funded by Riddet CORE.

We would like to invite you to participate in our study which aims to investigate the relationship between types of food you eat and the bacteria in the gut on bone density. Read through this information sheet carefully before making a decision on whether or not to participate. The information sheet will inform you on why we are doing this trial, whether you meet the study inclusion criteria, what is involved in the study, the risks and benefits of participating in the study and participants' rights. You are welcome bring along a family/whanau member or support person to each of your visits to the Massey University Human Nutrition Research Unit.

If you agree to participate in the study you will need to complete a Consent Form.

Why are we doing this trial?

Osteoporosis is a health problem in the elderly with hip and spine fractures occurring commonly after the age of 70. Low bone mineral density (BMD) is a risk factor for osteoporosis, with early detection allowing precautionary measures. There are many factors that increase the risk of osteoporosis and fractures including genetics, age, diet, physical activity, hormones, alcohol intake, smoking and body weight.

The type of food you eat is known to have effect on the bacteria in your gut status which in turn could affect your bone health especially in the postmenopausal years. Studying the patterns of New Zealand-type diet and its relationship with the gut and bone mineral density could give a valuable insight into the role of this diet in the gut and its effects on bone health.

If you are interested in taking part, please contact Lilian, Marlena or Anne who will be happy to discuss the project and answer your questions.

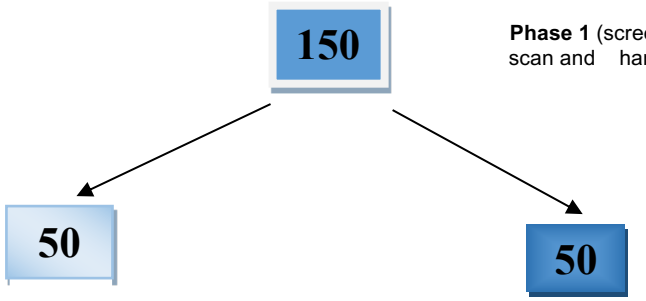
Contact details:

Lilian B. Ilesanmi-Oyelere
School of Food and Nutrition
Massey University
Private Bag 11222
Palmerston North
ext.84566
Telephone: [redacted]
e-mail: **bugsnbones2017@gmail.com**

Professor Marlena Kruger
School of Food and Nutrition
Massey University
Private Bag 11222
Palmerston North
Telephone: 06-951 7571
e-mail: m.c.kruger@massey.ac.nz

Ms Anne Broomfield
Research Technical Officer
Human Nutrition Research Unit
School of Food and Nutrition
Telephone: 06-356 9099
e-mail: a.m.broomfield@massey.ac.nz

Study design



Phase 1 (screening survey, DEXA scan and handouts)

Phase 2 (blood, urine and faecal sample collection for analysis)

Note:
Phase 1 - 150 participants sought after
Phase 2 - we will invite 100 people (2 groups of 50 each) from phase 1 to return for further investigation based on bone health

Study Inclusion Criteria

A total of 150 postmenopausal women 55 - 70 years are required for this study. Phase 1 data specifically bone health, will be used to select up to 100 women for phase 2 (for further investigation of their gut health and relationships).
To be able to participate in this study you need to meet the following criteria:

- Aged 55 - 70 years
- 5 years past menopause
- BMI between 18 and 30kg/m^2
- No significant weight loss or weight gain within the past year (i.e. 5% of your body weight)
- No food intolerances which cause gastrointestinal symptoms (i.e. lactose intolerance)
- Non-smoker
- Do not have a high intake of alcohol [(more than 2 standard drinks per day) i.e. 100 ml glass of table wine @ 12.5% alcohol = 1 standard drink or 330 ml can of beer @ 4% alcohol = 1 standard drink]

What are we going to measure?

PHASE 1
Questionnaires

- We will make some measurements and ask you questions about your diet, lifestyle and any physical activity or medications.
- Bone and heel scan for bone density measurement.
- You will be asked to fill in (at home) a 3-day diet diary (2 week days and a weekend), Food Frequency Questionnaire (FFQ) and the New Zealand Physical Activity Questionnaire short form (NZPAQ-SF).

Body measurements:
We will measure your height, weight, waist and hip circumference, and the length of some of your bones. These measurements will be conducted in private. Body weight will be measured using ordinary weighing scales (you will be asked to remove your shoes and outer clothes) and standing height will be measured using a wall measure and all other measurements will be made using a tape measure.

Bone scan
Measurement involves you lying down on a bed fully clothed, wearing surgical scrubs, on our bone scanning machine used for estimating body composition. With X-ray beams at two different energies it can estimate the difference between lean and fat tissue very accurately. We will also use the machine to assess your bones, to estimate the bone mineral density and the bone mineral content of your hip and femur. While no dose of radiation is harmless this dose is very low and unlikely to cause harm. The total effective dose of radiation to which you will be exposed to is 10.8 microsieverts (μSv), which is much lower than the range normally used in medical diagnostics. To place in perspective, the amount of radiation you are exposed to during a flight to

the United Kingdom return is 100 μSv and from a dental Xray 50 μSv. The procedure is quick, non-invasive and does not require anaesthetic. The room is private and you can enter the room in complete privacy.

The staff who do this are certificated to operate the machine and the scans will be assessed and approved by our consultant Radiologist. If your scan shows a T score of > 2.5 S.D below normal, you will be advised and a copy of the scan, the report from the radiologist and a letter provided to take to your GP to discuss if further investigation is necessary.

If you elect to receive the results of your bone scan and they show abnormalities, later if you seek life or health insurance you may be asked to disclose them by the insurer. Failure to disclose them could invalidate your insurance policy.

Heel scan
We will also take a quantitative ultrasound scan on your heel using an Ultrasound. This form of scanning is used as a tool for indication of bone density. The results will be used for comparison with the bone scan.

PHASE 2
Fasting blood samples
- You will visit the MedLab Palmerston North around 9am on a selected day having fasted since 9.00pm the night before.
- A qualified and experienced phlebotomist will take 25ml blood sample (about 5 tablespoons) to analyse bone markers of bone metabolism, vitamin D as well as lipid profile. Inflammatory markers will also be measured.

Urine samples
- You will be provided with a urine sample bottle for a urine sample. The urine samples will be collected for the measurement of bone bio-markers. Urine creatinine levels will also be measured.

Faecal samples
- You will need to collect a faecal sample and either bring it in or call for the sample to be picked up within a 30minutes time frame. We will provide equipment for you to collect this sample. The sample will be triple contained which means it will be stored hygienically.

Please note: The researchers will be storing samples for later study and determination of interactions between dietary pattern and risk of fractures on the understanding that I will be contacted for consent.

Time Involved

Phase 1 will take approximately 2 hours while phase 2 will take about 2 hours.
Individual time taken to fill in the questionnaires might vary but will be about 30 minutes for the 3-day diet diary and 20 minutes each for the baseline participants' questionnaire, food frequency questionnaire and physical activity questionnaire.

Will I get any financial compensation?

You will receive a **$20 petrol voucher for your participation in phase 1 and $30 grocery voucher for participating in the phase 2 of the study**.

What are the benefits and risks of taking part in this study?

Benefits
The benefits of being involved in this study is that you will receive information on your bone health i.e. the bone scan report and heel scan print-out.

If you are interested, you can also request to be sent information on your dietary intake analysis and body composition measurements.

You will also receive a summary of the main findings of the study.

Risks
As with all blood tests there may be some discomfort when the needle is inserted. You may also receive a bruise after the blood sample is taken, however this is unlikely. A certified and experienced phlebotomist will draw the blood.

We will also use the bone scan to assess your bones, to estimate the bone mineral density and the bone mineral content of your hip and femur. While no dose of radiation is harmless this dose is very low and unlikely to cause harm. The total effective dose of radiation to which you will be exposed to is 10.8 microsieverts (μSv), which is much lower than the range normally used in medical diagnostics. To place in perspective, the amount of radiation you are exposed to during a flight to the United Kingdom return is 100 μSv and from a dental Xray 50 μSv.

Who will see the information about me?

When you join the trial you will be given a number and thereafter all information will be filed with the code number, and stored in a locked filing cabinet accessed by the research team only. When information from all the volunteers has been pooled, and made anonymous, and used in the PhD Thesis. No names will be used, just the designated numbers.
All personal data will be destroyed at the end of the trial. Scientific data, filed on paper, will be shredded and electronic data will be deleted from our computer records and databases after 10 years. For the first 5 years it will be stored in a locked filing cupboard within a locked office. For the last 5 years it will be stored in a secure archive where all data is stored in boxes labelled by barcode only. It is accessible by nominated staff only, who require pin numbers for ID.

Should there be any abnormalities detected in your blood samples which MedLab will analyse, you will be referred to your General Practitioner. If anything untoward is found in any your tests Marlena will discuss it with you and asked whether you would like the results to be given to your medical practitioner or sent directly to you.

Compensation for Injury

If physical injury results from your participation in this study, you should visit a treatment provider to make a claim to ACC as soon as possible. ACC cover and entitlements are not automatic and your claim will be assessed by ACC in accordance with the Injury Prevention, Rehabilitation and Compensation Act 2001. If your claim is accepted, ACC must inform you of your entitlements, and must help you access those entitlements. Entitlements may include, but not be limited to, treatment costs, travel costs for rehabilitation, loss of earnings, and/or lump sum for permanent impairment. Compensation for mental trauma may also be included, but only if this is incurred as a result of physical injury.

If your ACC claim is not accepted, you should immediately contact the researcher. The researcher will initiate processes to ensure you receive compensation equivalent to that to which you would have been entitled had ACC accepted your claim from Massey University.

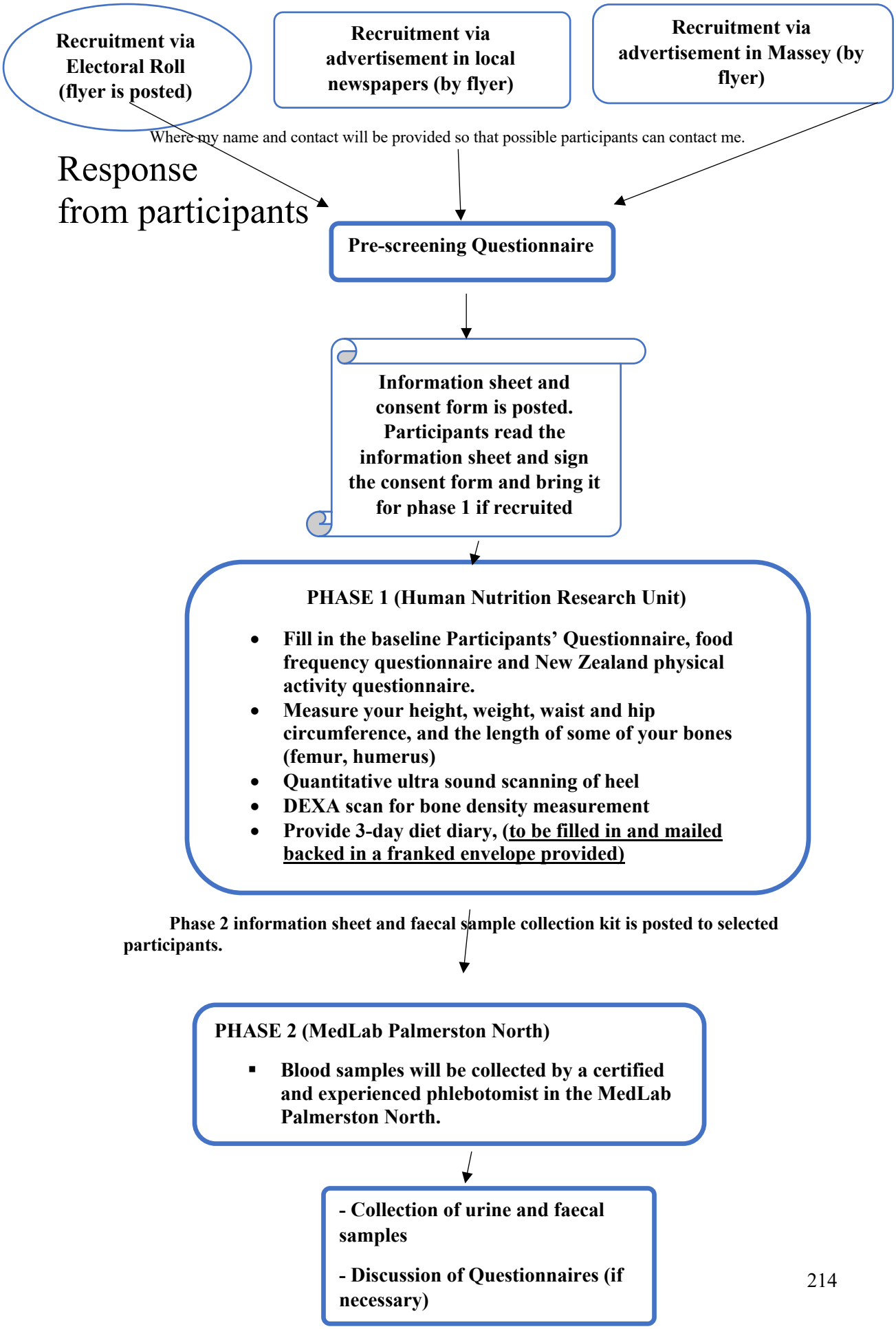
What are my rights?

We respect your rights to:
- decline to answer any particular question, and to withdraw from the study at any time
- withdraw from the study at any time
- ask further questions about the study that occur to you during your participation
- provide information on the understanding that it is completely confidential to the researchers. All information is collected confidentially, and it will not be possible to identify you in any reports that are prepared from the study
- be given access to a summary of the findings from the study when it is concluded.

Ethics Committee Approval

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 17/17. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 356 9099 x 85094, email humanethicsoutha@massey.ac.nz .

BUGS'n'BONES STUDY FLOW CHART



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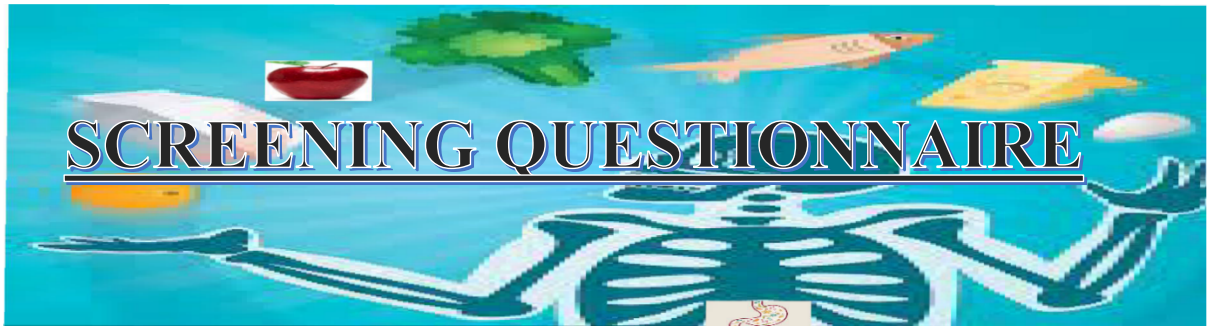

BUGS'n'BONES Study: The relationship between dietary patterns, bone bio-markers, gut microflora and bone density – A Cross-sectional Study

Thank you for expressing an interest in participating in our research project. To ensure you are eligible to participate in the research project we would appreciate it if you can answer the following questions.

If you have any comments or questions relating to the research project or the questionnaire, please feel free to contact **Lilian Ilesanmi-Oyelere** during working hours on [redacted] or email **bugsnbones2017@gmail.com**

OR

Professor Marlena Kruger during working hours on

(06) 951 7571

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Name: _____

Date of birth: _____

Date of menopause (last menstrual period):
_____

Do you smoke cigarettes (please tick)? Yes ☐ No ☐ Used to ☐

Do you drink alcohol (please tick)? Yes ☐ No ☐

If yes, how many standard drinks do you consume per week?
_____

(1 standard drink is 1 can/bottle of standard beer (330ml), 100ml wine or 30ml of spirits)

If yes, on how many occasions would you drink alcohol per week?
_____

Has your weight been stable over the past year (please tick)? Yes ☐ No ☐

If no, please record the amount of weight you have lost or gained? _____kg lost
_____kg gained

Current weight: ____ _____kg **Height**: _____cm

Have you been diagnosed with or experienced any of the following (tick for yes)?

Kidney disease	
Diabetes (type 1 or 2, or prediabetes)	
Bowel or gastrointestinal surgery	
Autoimmune disease (e.g. Coeliac disease, Rheumatoid arthritis, Multiple sclerosis)	
Liver disease	
Other (please specify):	

Thank you very much for taking the time to complete the screening questionnaire.

We will be in contact with you shortly.

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Bugs'n'Bones Study: The relationship between dietary patterns, bone biomarkers, gut microbiome and bone density

- I have read the Information Sheet and have had the details of the study explained to me.
- My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.
- I wish/do not wish to have data placed in an official archive.
- I consent to the researchers storing specimens for later determination of interactions between dietary pattern and risk of fractures on the understanding that I will be contacted for consent.
- I agree to participate in this study under the conditions set out in the Information Sheet.

I …….
hereby consent to take part in this study

Email address ……

Postal address ……

Day time phone number …….

General Practitioner ……..

Signature of volunteer……..

Date ……..

ID:

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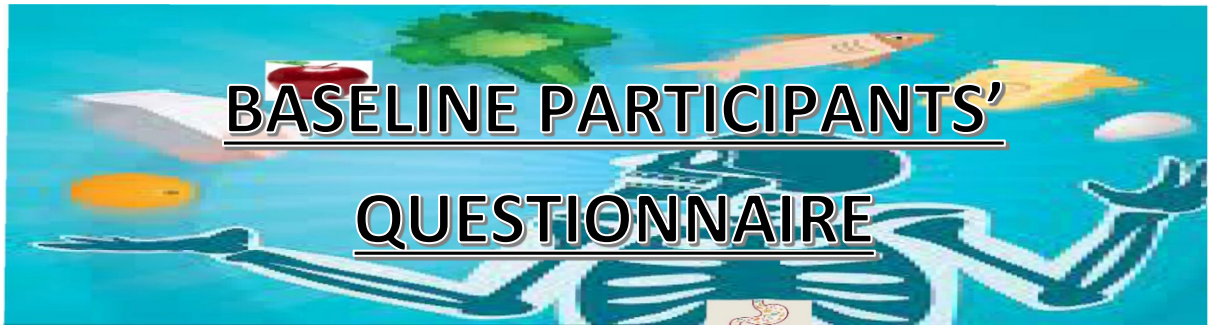

BASELINE PARTICIPANTS' QUESTIONNAIRE

BUGS'n'BONES Study: The relationship between dietary pattern, bone bio-markers, gut microflora and bone density – A Cross-sectional Study

Thank you for expressing an interest in participating in our research project. To ensure you are eligible to participate in the research project we would appreciate it if you can answer the following questions.

If you have any comments or questions relating to the research project or the questionnaire please feel free to contact **Lilian Ilesanmi-Oyelere** during working hours on [redacted] or email **bugsnbones2017@gmail.com**

OR

Professor Marlena Kruger during working hours on

(06) 951 7571

Date of birth: _____

Ethnicity (please tick all that apply):

NZ European ☐ Maori ☐ Samoan ☐ Cook Island Maori ☐

Tongan ☐ Indian ☐ Chinese ☐ Other ☐ please specify:

_____

Number of children _____

Highest level of education _____

How were you delivered Caesarean ☐ Normal ☐ Don't Know ☐

Do you drink alcohol (please tick)? Yes ☐ No ☐

If yes, how many standard drinks do you consume per week?

_____

(1 standard drink is 1 can/bottle of standard beer (330ml), 100ml wine or 30ml of spirits)

If yes, on how many occasions would you drink alcohol per week?

_____

Were you breastfed as an infant? Yes ☐ No ☐ Don't know ☐

Have you been diagnosed with or experienced any of the following (tick for yes)?

Osteoporosis	
Heart disease	
Stroke	
High cholesterol	
High blood pressure	
Cancer	
Inflammatory bowel disease	
Irritable bowel syndrome	
Food intolerance or allergies causing diarrhoea, bloating, cramping or constipation	
Long term diarrhoea or constipation	
Fractures	
If yes, how many?	
Other (please specify):	

Are you taking any medications (traditional or homeopathic) **or nutritional supplements?**

Type of medication/supplement	**Taking?** (please tick)	**If you have answered <u>YES</u> for any of the medication or supplement options please provide the below information**	
		Medication/supplement name	***Dose and frequency***
Antibiotics	Yes ☐ No ☐		
Blood pressure lowering	Yes ☐ No ☐		
Cholesterol lowering	Yes ☐ No ☐		
Vitamins	Yes ☐ No ☐		
Minerals	Yes ☐ No ☐		
Laxatives	Yes ☐ No ☐		
Metamucil or Benefibre	Yes ☐ No ☐		
Phloe or Kiwicrush	Yes ☐ No ☐		
Probiotics	Yes ☐ No ☐		
Prebiotics	Yes ☐ No ☐		
Antacids or anti-reflux	Yes ☐ No ☐		
Anti-inflammatory	Yes ☐ No ☐		
Other	Yes ☐ No ☐		

Have you consumed prebiotic or probiotic yoghurt, or fermented drinks or foods within the <u>past month</u> (i.e. Symbio probalance, Bio yoghurt, Yoplait Elivae, Activate, Bio farm organic, Yakult, Kefir, Sauerkraut, Kimchi, Kombucha) (please tick)?

Yes ☐ No ☐

If yes, please specify the product name and frequency of consumption:

_____

_____

____

Have you taken antibiotics within the <u>last 6 months</u> (please tick)?

Yes ☐ No ☐

If yes, please specify the name of the antibiotic and when you last took it:

_____

How often do you take antibiotics in <u>a year</u> (please tick)?

Once ☐ Twice ☐ 3 times ☐ 4 times or more ☐

Have you made any significant changes to your food intake over the <u>past year</u> (i.e. become vegetarian/vegan, stopped consuming gluten, dairy or sugar, increased your fruit and vegetable intake or increased/decrease the amount of food you are eating) (please tick)? Yes ☐ No☐

If yes, what changes to your food intake have you made?

_____

_____

____

Do you regularly experience any of the following (please tick all that apply)?

Abdominal pain ☐ Abdominal bloating ☐

Flatulence/wind ☐

If you experience abdominal pain, bloating or flatulence/wind is it mild (nagging/annoying)**, moderate** (strong negative influence on your daily living) **or severe** (disabling) (please tick the boxes that apply)?

	Absent	**Mild**	**Moderate**	**Severe**
Abdominal pain				
Abdominal bloating				
Flatulence/wind				

Thank you very much for taking the time to complete this questionnaire.

FOOD FREQUENCY QUESTIONAIRE

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ID:

EATING PATTERN & FOOD FREQUENCY QUESTIONNAIRE

1. Please describe your **usual** eating pattern (please mark one only):

	EATING PATTERN	Please tick one
	Eat a variety of foods, including animal products	
	Eat eggs, dairy products, fish and chicken but avoid other meats	
	Eat eggs and dairy products but avoid all meats and fish	
	Eat eggs but avoid dairy products, all meats and fish	
	Eat dairy products but avoid eggs, all meats and fish	
	Eat no animal products	
	Other – please specify:	

2. **On average**, how many servings of <u>fruit</u> (fresh, frozen, canned or stewed) do you eat per day?

Do not include fruit juice or dried fruit.

(a “serving” = 1 medium piece or 2 small pieces or ½ cup of stewed fruit)

	FRUIT Per Day	Please tick one
	I don’t eat fruit	
	Less than 1 per day	
	1 serving	
	2 servings	
	3 servings	
	4 or more servings	

3. **On average**, how many servings of vegetables (fresh, frozen, canned) do you eat per day?

Do not include vegetable juices.

(a “serving” = 1 medium potato/kumara **or** ½ cup of cooked vegetables **or** 1 cup of salad vegetables)

	VEGETABLES Per Day	Please tick one
	I don’t eat vegetables	
	Less than 1 per day	
	1 serving	
	2 servings	
	3 servings	
	4 or more servings	

4. **On average**, how many slices or rolls of <u>bread or toast</u> do you eat per day?

	BREADS Per Day	Please tick one
	I don't eat bread or toast *Chapter 9 Go to question 6*	
	Less than 1 per day	
	1 – 2	
	3 – 4	
	5 – 6	
	7 or more	

5. What type(s) of bread, rolls or toast do you eat most often?

(please only mark those you usually eat)

	BREADS Usual types	Please tick one
	Chapter 10 White	
	White – high fibre	
	Wholemeal or wholegrain	
	Other – please specify:	

6. Do you put butter or margarine on bread or crackers?

If no, go to question 7.

If yes, what type(s) do you use most often?

		Please tick
	Chapter 11 Butter	
	Unsalted butter	
	Butter and margarine blend	
	Low salt margarine	
	Polyunsaturated margarine eg “Miracle”, “Sunflower” etc	
	Reduced fat margarine eg “Slimarine”	
	“Praise” or “Olivio” margarine	
	Other – please specify:	

7. Do you usually eat breakfast cereal?

If no – please go to question 8

If yes – what breakfast cereal(s) do you have most often?

		Please tick one
	Chapter 12 Weetbix	
	Cornflakes or rice bubbles	
	Toasted muesli	
	Untoasted muesli	
	Special K	
	Ricies	
	All-Bran, San-Bran, Bran flakes, of Weetbix “Hi-Bran”	
	Puffed Wheat or mini wheats	
	Porridge	
	Other – please specify:	

8. Milk - do you use or drink any type of milk? Yes/No

If no – please go to question 9.

If yes, what type do you have most often?

	MILK	Please tick
	Standard, homogenised milk (dark blue top)	
	Trim milk (green top)	
	Super Trim	
	Skim milk or low fat milk powder	
	Semi-skimmed milk (light blue top)	
	Whole or powdered whole milk (silver top)	
	'Calci-Trim' (yellow top)	
	'Slim and Fit'	
	Soy milk	
	Other – please specify:	

9. Do you eat eggs?

If no – please go to question 10.

If yes – how many eggs do you usually eat **per week** (do not count eggs used in baking etc).

	EGGS Per Week	Please tick one
	Less than 1 per week	
	1 egg	
	2 eggs	
	3 eggs	
	4 eggs	
	5 or more eggs per week	

10. Do you eat pork, beef, mutton, hogget or lamb?

If no – please go to question 11.

If yes – do you trim any excess fat off these meats?

		Please tick one
	Always	
	Often	
	Occasionally	
	Never cut the fat off meat	

11. Do you eat chicken?

If no – please go to question 12.

If yes – do you remove the skin from chicken?

		Please tick one
	Always	
	Often	
	Occasionally	
	Never cut the fat off meat	

12. Do you eat meat or chicken fried or roasted in fat or oil?

If no – please go to question 13.

If yes – what types of fat or oil do you use most often?

		Please tick one
	Butter	
	Margarine	
	Lard or dripping	
	Olive oil or canola oil	
	Other oils e.g. sunflower, corn oil, safflower oil etc.	
	Don’t know	
	Other (please specify)	

13. Do you eat your vegetables fried or roasted in fat or oil?

If no – please go to question 14.

If yes – what type(s) of fat or oil do you use most often?

		Please tick one
	Butter	
	Margarine	
	Lard or dripping	
	Olive oil or canola oil	
	Other oils e.g. sunflower, corn oil, safflower oil etc.	
	Don’t know	
	Other (please specify)	

14. DAIRY FOODS

How often do you usually eat these foods or drinks?

Serving size of each food	Please tick one box for each food or drink.	**Never**	**Less than once a month**	**1-3 times a month**	**Once a week**	**3-4 times a week**	**5-6 times a week**	**Once a day**	**2 or more times a day**	
	Milk as a drink									
	Flavoured milk (e.g. milkshake, iced coffee)									
	Milk on breakfast cereals									
	Milk added to hot beverages made with water (e.g. coffee, tea, Milo)									
	Hot beverages made with milk (e.g. Milo, cocoa, hot chocolate drinks)									
	Cream or sour cream									
	Ice cream									
	Custard or milk based sauces									
	Yoghurt, plain or flavoured (including fromage frais)									
	Milk puddings (eg rice, semolina, instant)									
	Cream cheese									
	Cottage or ricotta cheese									
	Mozzarella, feta or camembert									
	Edam or gouda cheese									
	Colby, mild, tasty cheese, Cheddar									
	Brie, blue and other specialty cheeses									

15. BREADS & CEREAL FOOD

How often do you usually eat these foods?

Serving size of each food	Please tick one box for each food or drink.	**Never**	**Less than once a month**	**1-3 times a month**	**Once a week**	**3-4 times a week**	**5-6 times a week**	**Once a day**	**2 or more times a day**	
	Focaccia, bagel, pita or other specialty bread									
	Crumpet, croissant, waffle, doughnut, fruit- or iced-bun, scone, pikelet, muffin, slice of cake or similar									
	Savoury or dry biscuits, crispbread or crackers									
	Rice (brown or white)									
	Pasta eg spaghetti, ravioli, macaroni, noodles									
	Cooked porridge									

16. MEAT & FISH

How often do you usually eat these foods?

Serving size of each food	Please tick one box for each food or drink.	**Never**	**Less than once a month**	**1-3 times a month**	**Once a week**	**3-4 times a week**	**5-6 times a week**	**Once a day**	**2 or more times a day**	
	Beef, veal, hogget, lamb or pork dishes *include sausages, mince dishes (eg shepherds pie), mixed dishes (eg casserole, stir fry) and food eaten out (eg MacDonalds and takeaways)*									
	Chicken and other poultry dishes (eg turkey or duck)									
	Other meat eg venison, mutton bird									
	Bacon or ham, luncheon meats, salami or brawn, meat fillings in sandwiches									
	Liver (including pate)									
	Other offal (eg kidneys)									
	White fish dishes eg cod, roughy, etc *include battered fish, fish fingers, fish cakes etc*									
	Oily fish dishes eg sardine, tunas and salmon									
	Canned fish: please specify:									
	Shellfish and other sea food (eg mussels, oyster, paua, kina, pipis) or crab or prawns									

17. VEGETABLES (including fresh, frozen, canned)

How often do you usually eat these foods?

Serving size of each food	Please tick one box for each food or drink.	**Never**	**Less than once a month**	**1-3 times a month**	**Once a week**	**3-4 times a week**	**5-6 times a week**	**Once a day**	**2 or more times a day**	
	Root vegetables eg potato, kumara, turnip, swede, parsnips, carrots, beetroot– *boiled, mashed, baked, roasted*									
	Hot potato chips or kumara chips/ French fries/wedges									
	Peas, beans, sweets corn, mixed veg, lentils, baked beans									
	Green leafy vegetables eg kale, spinach, silver beet, puha, watercress, mustard greens, Taro leaf (eg palusami), Whitloof, Karengo (seaweed)									
	Brussels sprouts, cabbage, cauliflower, coleslaw, broccoli, broccoflower									
	Lettuce or green salad									
	Onions or leeks									
	Mushrooms									
	Tomatoes									
	Rhubarb									
	Sprouts (eg alfalfa, mung)									
	Soybeans, tofu, Taro									
	Green bananas (plantain)									
	Courgette/zucchini, marrow, cucumber, eggplant, squash, kamo kamo									
	Capsicum or peppers,									
	Spring onions									
	Asparagus									
	Avocado									

18. FRUIT (fresh, frozen, stewed or canned)

How often do you usually eat these foods?

Serving size of each food	Please tick one box for each food or drink.	**Never**	**Less than once a month**	**1-3 times a month**	**Once a week**	**3-4 times a week**	**5-6 times a week**	**Once a day**	**2 or more times a day**	
	Banana									
	Apple or pear									
	Oranges, mandarins, tangelos or other citrus fruit									
	Kiwifruit									
	Nectarine, peach, plum or apricot									
	Blackcurrants									
	Strawberries or other berries or cherries									
	Grapes									
	Raisins, sultanas, currants or other dried fruit (eg apricots, prunes, dates)									
	Mango, paw-paw, persimmon, feijoa, tamarillo, melon, pineapple or other fruit									
	Figs									

19. How often do you usually eat these foods?

Serving size of each food	Please tick one box for each food or drink.	**Never or rarely**	**3 times a month or less**	**1-2 times per week**	**3-6 times per week**	**1-2 times per day**	**3-5 times per day**	**6 or more times per day**	
	Mayonnaise								
	Low-calorie salad dressing								
	Tomato sauce								
	Gravy								
	White sauce/ cheese sauce etc								
	Coconut cream								

20. MISCELLANEOUS

How often do you usually eat these foods?

Serving size of each food	Please tick one box for each food or drink.	**Never**	**Less than once a month**	**1-3 times a month**	**Once a week**	**3-4 times a week**	**5-6 times a week**	**Once a day**	**2 or more times a day**	
	Sweet pies or sweet pastries									
	Other puddings or desserts (milk-based)									
	Biscuits -plain sweet, cream-filled or chocolate biscuits									
	Canned or packet soup (in winter)									
	Home-made soup (in winter)									
	Pizza									
	Muesli bars									
	Chocolate (including chocolate bars eg Moro bars)									
	Other confectionery									
	Jam, honey, marmalade or syrup									
	Peanut butter, other nut spreads									
	Vegemite or marmite									
	Nuts									
	Potato crisps, corn chips, Twisties etc									
	Meat pie, sausage roll or other savoury pastries.									
	Hamburger									

21. DRINKS – *Note that the possible categories have changed*

Serving size of each food	Please tick one box for each food or drink.	**Never or rarely**	**3 times a month or less**	**1-2 times per week**	**3-6 times per week**	**1-2 times per day**	**3-5 times per day**	**6 or more times per day**	
	Fruit juice eg fresh orange juice, Just Juice, Fresh-up, Robinson's, Rio Gold etc								
	Fortified juice: please specify type								
	Vegetable juice (eg tomato juice)								
	Fruit drink eg Choice, Rio Splice etc								
	Powdered drink (eg Raro, Vita-fresh)								
	Cordials/squashes including low calorie cordials								
	Carbonated drinks including low calorie drinks (eg Coke, lemonade, diet Sprite)								
	Sport's drinks (eg Gatorade, Powderade)								
	'Energy' drinks eg V, E2, Red Bull								
	Water (including unflavoured mineral water, soda water, tap water)								
	Coffee								
	Coffee – decaffeinated								
	Coffee substitute (eg Inka)								
	Milo, Cocoa, koko								
	Tea								
	Herbal tea								
	Soy beverages								
	Beer – low alcohol								
	Beer – ordinary								
	Red wine								
	White wine or champagne/sparkling wine								
	Wine cooler								
	Sparkling grape juice								
	Sherry or port								
	Spirits, liqueurs								

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ID:

Bugs'n'Bones Study

3 Day Food Record

Thank you very much for taking part in the Bugs'n'Bones Study. We are extremely grateful for your time, effort and commitment!

(2 week days and one weekend)

If you have any questions, please contact Lilian Ilesanmi-Oyelere on [redacted] *or email*

bugsnbones2017@gmail.com

All information in this diary will be treated with the strictest confidence. No one outside the study will have access to this.

Please post this diary in the franked envelope when you have completed the information.

Reminders for your next appointments

- Bring this diary with you to your next appointment

- Wear comfortable, casual clothes including a top with either short-sleeves on no sleeves.

- Your appointment will last approximately 2 hours so bring something along to entertain yourself with – your laptop and personal DVDs, a book, magazines, iPod or study notes.

Second Appointment

Date
Time

If for any reason you are going to be unable to come for this appointment at the scheduled time and/or day, please let us know as soon as possible.

Email: **bugsnbones2017@gmail.com**

Phone: *Lilian Ilesanmi-Oyelere on* [redacted]

3 day food diary - What to do?

- Record all that you eat and drink on the following dates (please include a weekend):

_____

_____

_____

- If possible record food at the time of eating or just after – try to avoid doing it from memory at the end of the day.

- Include all meals, snacks, and drinks, even tap water.

- Include anything you have added to foods such as sauces, gravies, spreads, dressings, etc.

- Write down any information that might indicate size or weight of the food to identify the portion size eaten.

- Use a new line for each food and drink. You can use more than one line for a food or drink. See the examples given.

- Use as many pages of the booklet as you need.

Describing Food and Drink

- Provide as much detail as possible about the type of food eaten. For example **brand names and varieties / types** of food.

General description	Food record description
Breakfast example – cereal, milk, sugar	1 cup Sanitarium Natural Muesli 1 cup Pam's whole milk 1 tsp Chelsea white sugar
Coffee	1 tsp Gregg's instant coffee 1 x 200ml cup of water 2 Tbsp Meadow fresh light green milk
Pasta	1 cup San Remo whole grain pasta spirals (boiled)
Pie	Big Ben Classic Mince and Cheese Pie (170g)

- Give details of all the **cooking methods** used. For example, fried (sort of oil/fat used), grilled, baked, poached, boiled…

General description	Food record description
2 eggs	2 size 7 eggs fried in 2tsp canola oil 2 size 6 eggs (soft boiled)
Fish	100g salmon (no skin) poached in 1 cup of water for 10 minutes

- When using foods that are cooked (eg. pasta, rice, meat, vegetables, etc), please record the **cooked portion** of food.

General description	Food record description
Rice	1 cup cooked Jasmine rice (cooked on stove top)
Meat	90g lean T-bone steak (fat and bone removed)
Vegetables	½ cup cooked mixed vegetables (Wattie's peas, corn, carrots)

- Please specify the **actual amount of food eaten** (eg. for leftovers, foods where there is waste)

General description	Food record description
Apple	1 x 120g Granny Smith Apple (peeled, core not eaten – core equated to ¼ of the apple)
Fried chicken drumstick	100g chicken drumstick (100g includes skin and bone); fried in 3 Tbsp Fern leaf semi-soft butter

- Because we are especially interested in your calcium intake, please take care to list **all** the milk you consume, and record what type of milk it was.

General description	Food record description
hot chocolate	I x cup hot chocolate made with Cadbury's powder and 150 mls Anchor Calcitrim milk, 100 ml hot water. No sugar

- **Record recipes** of home prepared dishes where possible: record how many the recipe fed and the proportion of the dish you ate. There are blank pages for you to add recipes or additional information.

Recording the amounts of food you eat

It is important to also record the quantity of each food and drink consumed. This can be done in several ways.

- By using household measures – for example, cups, teaspoons and tablespoons. eg. 1 cup frozen peas, 1 heaped teaspoon of sugar.

- By weight marked on the packages – eg. a 425g tin of baked beans, a 32g cereal bar, 600ml Coke

- Weighing the food – this is an ideal way to get an accurate idea of the quantity of food eaten, in particular for foods such as meat, fruits, vegetables and cheese.

- For bread – describe the size of the slices of bread (eg. sandwich, medium, toast) – also include brand and variety.

- Using comparisons – eg. Meat equal to the size of a pack of cards, a scoop of ice cream equal to the size of a hen's egg.

- Use the food record instructions provided to help describe portion sizes.

General description	**Food record description**
Cheese (Edam)	1 heaped tablespoon of grated cheese 1 slice cheese (8.5 x 2.5 x 2mm) 1 cube cheese, match box size Grated cheese, size 10B

- If you go out for meals, describe the food eaten in as much detail as possible.

- ***Please eat as normally as possible - don't adjust what you would normally eat just because you are keeping a diet record and be honest! Your food record will be identified with a number rather than your name.***

Example day

Time food was eaten	**Complete description of food (food and beverage name, brand, variety, preparation method)**	**Amount consumed (units, measures, weight)**
Example 7:55am	Sanitarium weetbix	2 weetbix
" "	Anchor Blue Top milk	150ml
" "	Chelsea white sugar	2 heaped teaspoons
" "	Orange juice (Citrus Tree with added calcium – nutrition label attached)	1 glass (275 ml)
10.00am	Raw Apple (gala)	Ate all of apple except the core, whole apple was 125g (core was ¼ of whole apple)
12.00pm	Home made pizza (recipe attached)	1 slice (similar size to 1 slice of sandwich bread, 2 Tbsp tomato paste, 4 olives, 2 rashers bacon (fat removed), 1 Tbsp chopped spring onion, 3 Tbsp mozzarella cheese)
1.00pm	Water	500ml plain tap water
3.00pm	Biscuits	6 x chocolate covered Girl Guide biscuits (standard size)
6.00pm	Lasagne	½ cup cooked mince, 1 cup cooked Budget lasagne shaped pasta , ½ cup Wattie's creamy mushroom and herb pasta sauce, ½ cup mixed vegetables (Pam's carrots, peas and corn), 4 Tbsp grated Edam cheese
6.30pm	Banana cake with chocolate icing (homemade, recipe attached)	1/8 of a cake (22cm diameter, 8 cm high), 2 Tbsp chocolate icing
" "	Tip Top Cookies and Cream ice cream	1 cup (250g)
7.30pm	Coffee	1 tsp Gregg's instant coffee 1 x 300ml cup of water

		2 Tbsp Meadow fresh blue top milk 2 tsp sugar

Date_____ DAY 1

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date_____ **DAY 1 continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recipes (Day 1)

Recipes (Day 2)

Date_____ DAY 2

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date_____ **DAY 2 continued**

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date_____ DAY 3

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Date_____ DAY 3 continued

Time food was eaten	Complete description of food (food and beverage name, brand, variety, preparation method)	Amount consumed

Recipes (Day 3)

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Bugs'n'Bones study

Phase 2: Information for collection of blood, urine and faecal samples

Thank you very much for participating in phase 1 of this study and thank you for deciding to take part in phase 2.

Please visit us for fasting blood collection as well as the MedLab.

Urine and faecal sample would need to have been collected in the morning.

- Please visit HNRU (School of Food and Nutrition) fasting on **Friday, February 9th** early in the morning.
- Kindly visit the MedLab at your convenience with the MedLab Central slip.
- Drink lots of water the day before the blood collection day.
- Please kindly come fasted on the day. ***Do not eat anything after 9pm the night before.*** You may drink water. Do not eat breakfast or drink anything other than a small amount of water on the morning of your appointment.
- Wear comfortable, casual clothes including sleeve-less or short-sleeved top.
- Your appointment will last approximately 20-30 minutes.

- On the day of blood collection please collect early morning **Fasting midstream urine sample and faecal (stool) sample** into the urine and stool sample container provided. Otherwise, **please collect the faecal sample the night before the appointment and freeze**.

- Please bring in the fasting midstream urine and faecal sample to Massey University Human Nutrition Research Unit (HNRU) located in the School of Food and Nutrition.

- **You will receive a voucher for your participation when you come in to the HNRU. Thank you so much.**

Email: **bugsnbones2017@gmail.com**

Phone or text: *Lilian Ilesanmi-Oyelere on* [redacted]

Ethics Committee Approval

This project has been reviewed and approved by the Massey University Human Ethics Committee: Southern A, Application 17/17. If you have any concerns about the conduct of this research, please contact Dr Lesley Batten, Chair, Massey University Human Ethics Committee: Southern A, telephone 06 356 9099 x 85094, email humanethicsoutha@massey.ac.nz .

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Consent Form

The relationship between dietary patterns, bone biomarkers, gut microbiome and bone density- Phase 2

- *I have read the Information Sheet and have had the details of the study explained to me.*
- *My questions have been answered to my satisfaction, and I understand that I may ask further questions at any time.*
- *I wish/do not wish to have data placed in an official archive.*
- *I agree to participate in this study under the conditions set out in the Information Sheet.*

I ……..……..

hereby consent to take part in this study

Email address ……

Postal address ……

Day time phone number ……..

General Practitioner ……...

Signature of volunteer……...

Date ……...

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BUGS'n'BONES Study: The relationship between dietary patterns, bone biomarkers, gut microbiome and bone density

Please kindly read carefully

If you regularly have a bowel motion in the morning, please collect the fasting urine and faecal sample on the morning of your visit.

Once the faecal sample has been collected, please bring this sample to the Massey University Human Nutrition Research Unit or freeze. Otherwise please call Lilian on [redacted] **immediately** for collection. Thank you.

You have been provided with the following:

- A pair of disposable gloves
- A urine sample container
- A styrofoam rectangular container
- A stool sample container
- Anaerobic bag
- Anaerobic sachet
- A cooler bag
- A freezer pack
- A brown paper bag

Instructions:

1. Use the **disposable gloves** while collecting the urine and faecal sample.
2. Please put the sterile **freezer pack** in the freezer to become frozen.

3. To obtain the urine sample please collect a clean-catch **midstream fasting urine sample** into the **urine sample container** to at least ½ full. Ensure the lid on the **urine sample container** is sealed and secure in its bag.

4. To obtain the faecal sample (please try to fill the **stool sample container** to at least a 1/3 full):
    a. Catch the faecal sample using the **styrofoam rectangular container** (lined with toilet paper) before it reaches the toilet water. Use the **scoop on the lid** to transfer some of the faecal sample to the **stool sample container** (be careful not to contaminate the sample with any urine).

    OR

    b. Void the faecal sample straight into the **stool sample container** (be careful not to contaminate the sample with any urine).
5. Ensure the lid on the **stool sample container** is secure and then seal the **stool sample container** in the **anaerobic bag** with the **anaerobic sachet** inside (remove the tinfoil outer packaging to reveal the white anaerobic sachet before putting the anaerobic sachet in the anaerobic bag). **Disposable gloves** can be removed at this point.
6. The **anaerobic bag** can then be put into the **cooler bag** prior to putting it into the freezer. The faecal sample is now triple contained and can be handled safely.
7. Excess faeces can be disposed of down the toilet and any used items can be put into the **brown paper bag** provided.
8. The **brown paper bag** can then be disposed of in the rubbish.
9. When transporting the urine and faecal sample to the Massey University Human Nutrition Research Unit, put the frozen **freezer pack** into the **cooler bag** with the contained urine and faecal sample to ensure it stays cool. Thank you