Interactions of Vitamin D and Calcium on Bone Turnover, Quantity and Quality in Humans

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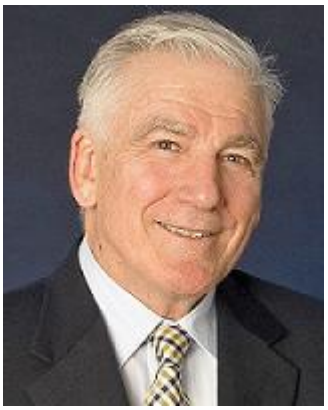
This thesis is dedicated to



Professor Howard A. Morris

A dynamic scientist, a great mentor, an inspiring leader,

a thoroughly good person with a contagious smile

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Abbreviations

$1,25(OH)_2D$	1,25-Dihydroxyvitamin D3
25(OH)D	25-Hydroxyvitamin D
AIHW	Australian Institute for Health and Welfare
BMD	Bone Mineral Density
BMU	Basic Multicellular Unit
Bone ALP	Bone-Specific Alkaline Phosphatase
BS/BV	Bone Surface/Bone Volume
BTM	Bone Turnover Marker
CaSR	Calcium Sensing Receptor
CI	Confidence Interval
CTX-1	C-terminal cross-linking telopeptide of type I collagen
CYP27B1	25-hydroxyvitamin D3 1α-hydroxylase
CYP24A1	25-hydroxyvitamin D-24-hydroxylase
CRP	C-Reactive Protein
DBP	Vitamin D binding protein
DXA	Dual-Energy X-ray Absorptiometry (DXA)
EAR	Estimated Average Requirement
FGF-23	Fibroblast Growth Factor-23
FRAX	Fracture Risk Assessment Tool
GM-CSF	Granulocyte Macrophages Colony-Stimulating Factors
IFCC	International Federation of Clinical Chemistry
IGF-1	Insulin like Growth Factor
IOF	International Osteoporosis Foundation
IOM	Institute of Medicine
HR-pQCT	High resolution Peripheral Quantitative Computed Tomography
M-CSF	Macrophages Colony-Stimulating Factors
Micro CT (μCT)	Micro-Computed Tomography
MWT	Mean Wall Thickness
NTFS	Newcastle Thousand Families Study

OPG	Osteoprotegerin
OR	Odds Ratio
PTH	Parathyroid Hormone
RANK	Receptor Activator of Nuclear factor Kappa-B
RANK-L	Receptor Activator of Nuclear factor Kappa-B ligand
RCT	Randomized Controlled Trial
RDI	Recommended Dietary Intake
RR	Risk Ratio
SAT	Subcutaneous Adipose Tissue
TBS	Trabecular Bone Score
VDR	Vitamin D Receptor
VAT	Visceral Adipose Tissue
WHI	The Women's Health Initiative Study
WC	Waist Circumference
WG-BMA	Working Group for Standardization of Bone Marker Assays

Abstract

Although vitamin D and calcium are important nutrients for the maintenance of skeletal health, their relative roles in preventing fragility fractures in bone is currently an unresolved and difficult debate. Contributing to the uncertainty for the role of vitamin D in bone, is a current lack of understanding of the cellular mechanisms by which vitamin D affects bone health. For calcium, while the role of calcium nutrition and bone health is relatively understood, the interaction of calcium with vitamin D and other biological factors such as body composition is less well understood. These often-unacknowledged gaps in knowledge, have led to over-simplification of the debate, especially in the area of clinical nutrition studies. The central aim of this thesis was to investigate some of these knowledge gaps. The studies presented here provides evidence for the cellular mechanisms through which serum 25 hydroxy vitamin D levels (25(OH)D) affects bone turnover, structure and quality. This thesis also investigates the complex interplay between central fat and bone health and the role of calcium supplementation in fat-bone association.

The first aim of this thesis was to investigate the impact of vitamin D metabolites (1,25 di-hydroxy vitamin D_3(1,25$(OH)_2$D) and 25(OH)D), parathyroid hormone levels (PTH) and bone turnover markers on femoral bone structure in bone biopsies taken from elderly orthopaedic patients. The central discovery of this study was that only serum 25(OH)D levels were strongly and independently associated with measures of bone quality, including mean wall thickness (MWT) and bone surface: bone volume ratio (BS/BV). Of significant relevance was the observation that serum 25(OH)D levels beyond that is required for renal 1,25$(OH)_2$D synthesis, continues to be associated with improvements in MWT and BS/BV. Furthermore, higher expression of bone 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1) and lower expression of 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) significantly contributed to the association of serum 25(OH)D levels and lower BS/BV ($p = 0.001$). These data demonstrate that serum 25(OH)D is an independent positive predictor of micro-structural and bone formation measures and provide a potential mechanism by which higher vitamin D status may improve bone quality independent of bone quantity.

The second aim of this thesis was divided in two studies. In first study, associations between visceral adipose tissue (VAT) and bone remodelling markers, total bone mineral density (BMD), 25(OH)D, 1,25$(OH)_2$D and PTH levels in healthy post-menopausal women were examined. The second study was to examine the acute effects of dietary calcium and a calcium supplement on the bone resorption marker, C-terminal cross-linking telopeptide of type I collagen (CTX-1) levels

in post-menopausal women with varying body mass index (BMI) and VAT levels. To accomplish these aims, a randomized cross-over study was conducted in healthy postmenopausal women with varying VAT and BMI levels (BMI < 25 kg/m^2 or > 27 kg/m^2). Each week, fasting participants were randomized to receive a single oral dose of either milk (1000 mg calcium), calcium carbonate tablet (1000 mg calcium) and fruit juice (no calcium, control). Data from these studies demonstrated that women in the highest VAT quartile had significantly lower CTX-1 levels, 25(OH)D levels and osteocalcin levels and higher PTH levels and BMD content, suggesting altered bone metabolism. While VAT positively associated with BMD, after controlling for BMI, VAT was a negative predictor of BMD. Despite these baseline differences, all women responded almost equally to dietary calcium and calcium carbonate with respect to suppression of CTX-1 levels over the 5 hours post-consumption. These data provide evidence for the role of VAT on bone markers in postmenopausal women and suggest that a 1000 mg calcium dose from both dietary source and a supplement will be beneficial in suppressing bone resorption in obese post-menopausal women.

Declaration of Authorship

This thesis presents work carried out by myself and does not incorporate without acknowledgment any material previously submitted for a degree or diploma in any university to the best of my knowledge and it does not contain any materials previously published or written by another person except where due reference is made in the text; and all substantive contributions by others to the work presented, including jointly authored publications is clearly acknowledged.

I give my consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.



DEEPTI K SHARMA
14th April 2020

Acknowledgments

"Gurur Brahma, Gurur Vishnuh, Gurur Devo Maheshvarah, Gurureva Param Brahma, Tasmai Shri Guarave Namah"

Adi Shankaracharya

Meaning: This is a shloka from Hindu scriptures which offers obeisance to the "Guru" or the "teacher" or the "supervisor" who takes us from *ignorance* to *knowledge*.

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Publication Arising

Elevated serum 25-hydroxyvitamin D levels are associated with improved bone formation and micro-structural measures in elderly orthopaedic patients. Deepti K Sharma, Rebecca K Sawyer Thomas S Robertson, Roumen Stamenkov, Lucian B Solomon, Gerald J Atkins, Peter M Clifton, Howard Morris, Paul H Anderson, *J Clin Med 2019 Nov;8(11):1988* (invited publication).

Publication Ready for Submission

Visceral fat is a negative determinant of bone health in post-menopausal women. Deepti K Sharma, Paul H Anderson, Peter M Clifton, Howard Morris (to be submitted to Nutrients, special issue Redefinition of OBESITY in the Context of Visceral and Subcutaneous Fat and Their Impact on Sarcopenia and Osteoporosis).

Acute effects of calcium supplementation on CTX-1 suppression in post-menopausal women with high and low visceral fat- a randomized cross-over study. Deepti K Sharma, Paul H Anderson, Peter M Clifton, Howard Morris.

Presentation Arising

International Conferences

Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA. *Bone-Derived 1,25-dihydroxyvitamin d is a determinant of trabecular bone structure in hip fracture patients*. International Congress in Vitamin D, March 2017, Orlando, FL, USA.

Sharma DK, Clifton PM, Anderson PH, Morris HA, *Autocrine actions of Vitamin D*, IOF-IFCC BWG meeting, Greece, 2017.

Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA. *Bone-Derived 1,25-dihydroxyvitamin d is a key determinant of trabecular bone structure in hip fracture patients*. ECTS PhD training program, July 2017, Paris.

Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA. *Bone CYP27B1, CYP24A1 AND Serum 25(OH)D are key positive factors for trabecular bone strength in hip fracture patients*. American Society of Bone and Mineral Research Conference, September 2017 Colorado, USA, 2017. no:FR0054. Plenary session

Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA. *Serum 25-hydroxyvitamin D and its metabolism in bone tissue is associated with improved bone quality in elderly hip fracture patients*. World Congress of Osteoporosis, April 2018, Krakow. Abstract ID 534.

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Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA. *Bone-derived 1,25(OH)$_2$D is a key determinant of trabecular bone structure in hip fracture patients*. Australia & New Zealand Bone and Mineral Society, Plenary session, Brisbane, Queensland, Australia, June 2017.

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Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA**.** *Vitamin D and its metabolism is directly associated with improved bone quality in elderly patients*. 10th Asia Pacific Conference on Clinical Nutrition, Adelaide, November 2017. Abstract no 280**.**

Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA. *Vitamin D and its metabolism is directly associated with improved bone quality in elderly patients.* Outstanding abstract presentation at Australian and New Zealand Society for Sarcopenia and Frailty Research, No:0053, Adelaide, November 2017.

Sharma DK, Sawyer R, Stamenkov R, Robertson T, Stapledon C , Atkins GJ, , Solomon LB , Clifton PM, Anderson, PH., Morris HA. *Bone Metabolism of Vitamin D is Associated with Improved Bone Quality in Elderly Hip Fracture Patients*. 10th Clare Valley Bone Meeting, South Australia, March 2018.

Sharma DK, Anderson PH, Clifton PM, Morris HA. *Visceral Fat alters Bone Health in Post-Menopausal Women but Acute Response to Calcium is not Affected*. Australia & New Zealand Bone and Mineral Society, Darwin, October 2019.

Sharma DK, Anderson PH, Clifton PM, Morris HA. *Obesity lowers Bone Turnover but Does not Alter the Response to Dietary Calcium in Post-Menopausal Women*. 43rd Nutrition Society of Australia, Annual Scientific Meeting, Newcastle, December 2019.

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Sharma DK, Robertson T, Stamenkov R, Stapledon CJM, Atkins GJ, Solomon LB, Clifton PM, Anderson PH, Morris HA. *Vitamin D and its Metabolism in Bone are Required for Improved Bone Microarchitecture*. School of Pharmacy and Medical Science Symposium, University of South Australia, Adelaide, June 2017.

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Awards Arising

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Chapter 1: Current Knowledge on Calcium and Vitamin D

'To raise new questions, new possibilities, to regard old problems from a new angle, require creative imagination and marks real advance in science'.

Albert Einstein

1.1 Introduction

Calcium and vitamin D play an essential role in maintaining bone health throughout the life cycle. Maximizing bone mineral mass acquisition during childhood and adolescence, preventing premature loss of bone mass and maintaining healthy skeleton in adult years and prevention of osteoporosis in elderly are the primary strategies to maintain bone health across life stages and calcium and vitamin D status are known to influence each of these strategy [1-5]. Genome-wide association studies have identified 70 loci associated with bone mineral density (BMD) or fracture risk [6] and twin studies suggest that up to 60-80% of peak bone mass acquisition is genetically determined [7, 8]. The remaining 20-40% is likely to be modulated by environmental factors such as calcium and vitamin D levels along with other nutrients and physical activity [7, 9, 10]. Regular intake of dietary calcium, vitamin D, dairy products, good quality protein along with weight- bearing physical activity, such as jumping helps to attain genetic potential for peak bone mass [8, 11]. In addition, there is emerging evidence on the role of other nutrients such as vitamin C, vitamin K, phosphorus, magnesium, manganese, zinc and copper in cellular processes involved with bone mass acquisition [2, 12]. It is predicted from epidemiological studies that a 10% increase in peak bone mass corresponds to a gain of one standard deviation in BMD in adulthood and a 50% reduction in osteoporotic fracture risk [7, 8, 11]. After the attainment of peak bone mass until the onset of age-related bone loss (i.e. 20-60 years, mostly), calcium alone or in combination with vitamin D may play a critical role in preventing premature loss of bone mass and delaying or preventing onset of osteoporosis [1, 13, 14]. Adequate calcium and vitamin D supplementation play an important role in preventing or delaying age related loss of bone mass [15-19] and has been shown to reduce the risk of fractures in at-risk populations and when compliance is at least 80% [18, 20-25].

Despite a large body of evidence, several uncertainties continue to persist with regards to role of calcium and vitamin D supplementation for bone health and whether they play a role in preventing fractures [26-29]. While numerous studies have demonstrated a relationship between vitamin D status and bone health, there is also considerable disagreement regarding optimal levels required for musculoskeletal health [27, 30-37].

A greater understanding of underlying cellular and molecular mechanism through which vitamin D and calcium act on bone in at-risk population would shed some light on the prevailing controversies and contribute towards bridging some of the knowledge gaps in the field. Hence, this thesis was undertaken to investigate mechanisms by which vitamin D and calcium improve bone health in two cohorts- elderly people and obese post-menopausal women, respectively.

This chapter provides a brief overview of the current state of knowledge in the field of calcium and vitamin D. First, bone physiology and physiological control of bone remodelling is discussed. Next, assessment of bone turnover and clinical usefulness of bone markers are discussed. This is followed by an in-depth discussion on a state of high bone turnover- i.e. *osteoporosis,* its pathology, assessment methods and the role of calcium and vitamin D supplementation in bone turnover, quantity and fracture risk. This is followed by aim 1 and hypothesis. Next, a state of low bone turnover i.e. *obesity* and its impact on skeletal health and fracture risk is discussed, concluding with aim 2 and hypothesis.

1.2 Bone Physiology

Bone is a best example of the principle that *form follows function*. Bone provides structural support for the entire body yet is light to enable mobility, is responsible for blood cell production and locomotion; acts as a reservoir for calcium, phosphate, amino acids, and bicarbonate; provides protection to interior organs; and is a source of hematopoietic stem cells [38]. Metabolic functions of bone include secretion of hormones that regulate both mineral and energy metabolism. To fulfil these functions, the bone has cortical and trabecular compartments [38, 39]. Trabecular or cancellous bone is composed of interconnecting rods and plates that form a lattice-like network with many pores. Cortical bone is compact and dense and surrounds the trabecular structures. Approximately, 80% of the bone mass is cortical bone and around 20% is trabecular bone [38, 39]. The two bones differ from each other in composition, location, function and metabolic activity [40]. Both cortical and trabecular bone are composed of osteons. Cortical bone is found in the diaphysis of the long and short bones of the extremities and surrounds the trabecular bone of the vertebral body, at the ends

of long bones, in the iliac crest and in the skull. Trabecular bone is found primarily in the metaphysis of the long bones, as well as in the vertebrae, ribs, and iliac crest [39]. Cortical osteons are called haversian systems which create a porosity of about 3-5%, although this increases with age and with alterations in bone microarchitecture due to osteoporosis [38]. Its function is to provide support and protection [39]. Cortical bone has an outer periosteal surface and inner endosteal surface. Trabecular osteons are called packets. A fifth of the trabecular volume is composed of bone and the remaining space is filled with marrow and fat, which makes it highly porous (30-90% porosity). The trabecular bone is composed of plates and rods of bone which comprises about 25-30% of the total tissue volume, with the remainder being marrow space. As they are adjacent to the marrow cavity, trabecular bone can derive their blood supply from it and hence do not need nor do they contain a central vascular channel. Trabecular bone derives its primary mechanical benefit from its architecture, which provides structural support without increasing the weight of the entire bone. Because of its location within the marrow cavity, trabecular bone by itself is not an efficient weight-bearing structure and provides a means for the bone to funnel the stresses imposed on it to cortical bone [38]. The ratios of cortical to trabecular bone varies between different bones and skeletal sites. The cortical to trabecular bone ratio in the vertebra is 25:75, 50:50 in the femoral head and 95:5 in the radial diaphysis [39]. Both cortical and trabecular bone are independent contributors to bone strength and fracture risk [41, 42].

1.3 Bone Remodelling and Turnover

Bone remodelling is an important process that continuously renews cortical and trabecular bone compartment with an annual turnover of about 10% of the adult skeleton [39]. Targeted bone remodelling assists in adjusting the bone architecture to meet altering mechanical needs and helps to repair micro-damages in bone matrix, thus preventing the accumulation of old bone [39, 43-45]. In the young skeleton, when the cycle is completed, the amount of bone formed is equal to the amount of bone resorbed. The process of bone remodelling takes place in a coordinated fashion and is under the control of local and systemic factors that together contribute to bone homeostasis [46]. An imbalance between bone resorption and formation results in untargeted bone

remodelling such as in metabolic bone diseases like osteoporosis [38, 44, 45]. A complete remodelling cycle takes 4-6 month, although this can be highly altered in disease state [47]. The duration of a remodelling cycle is not evenly divided between resorption and formation. Osteoclasts typically resorb bone for 3-6 weeks, with the remainder of the cycle comprising of bone formation [47]. Remodelling of bone continues throughout life and skeletal tissue is replaced every 10 to 12 years on an average [38]. Skeletal remodelling can be triggered by multiple factors: mechanical forces, bone micro-damage, hormonal alterations, sex hormone deficiency, adipokines, responses to change in mineral supply, etc. The key regulators of bone remodelling are discussed in section 1.3.3. Additional factors which influence remodelling are listed in table 1.1.

Table 1. 1 Factors influencing bone remodelling

Source	**Nature of effect**
Circadian rhythm	Bone resorption markers show highest values in the second half of night and early morning; lowest values in afternoon and evening[48].
Fasting status	Feeding results in a 20% decrease in bone resorption marker, carboxy-terminal cross-linking telopeptide of type I collagen (CTX-1) levels mediated through gut hormones [49].
Diet	Calcium supplementation or dietary calcium intake results in acute and chronic decline in CTX-1 levels [50-52].
Sex	Older women have higher bone remodelling marker levels than older men [48].
Physical activity	The extent and type of exercise can influence the levels of BTMs especially bone formation markers [53].
Stage of menstrual cycle	During the luteal phase, small decreases in bone resorption and increases in bone formation are observed [48].
Age	Aging is associated with increase in bone markers both in men and women [48].
Ethnicity	Ethnicity is known to affect the levels of BTMs [54].
Concomitant medication	BTMs may be decreased (glucocorticoids) or increased(anticonvulsants) [48].
Concomitant disease	Certain diseases can affect the levels of BTMs such as diabetes results in a reduction in both formation and resorption markers [55].
Pregnancy and lactation	The levels of bone remodelling markers increase during pregnancy and lactation [48]

The remodelling process requires coordinated cellular activities between all three bone cells, osteoclasts, osteoblasts and osteocytes to maintain bone homeostasis. Together, these cells form temporary anatomical structures, called basic multicellular

units (BMU) to accomplish bone remodelling. The different bone cells and their functions are briefly discussed below.

1.3.1 Major Cell Types Involved in Bone Remodelling

Osteoblast and bone formation: Osteoblasts are single nucleated cells originating from mesenchymal stem cells (MSC) known as osteoprogenitor cell [39]. Functionally, the osteoblasts are responsible for production of the organic matrix composed of proteins and polysaccharides called osteoid which subsequently mineralizes. Many growth factors such as insulin-like growth factors (IGF-1), members of transforming growth factor beta (TGF-β) such as bone morphogenetic protein (BMP) [56] affect bone formation. Some evidence exists to suggest that osteoblasts, under the influence of parathyroid hormone (PTH) and local cytokines such as receptor activator of the nuclear factor kappa-B receptor ligand (RANK-L), colony-stimulating factors (CSF) and interleukin-6 (IL-6) and interleukin-11 (IL-11) can activate and affect osteoclast recruitment to initiate a bone remodelling cycle [46, 47, 57]. Products of osteoblasts are used to monitor the rate of bone formation and are discussed in section 1.3.4.

Osteoclast and bone resorption: Osteoclasts are multinucleated cells originating from hematopoietic cell lineage and are the principle cells responsible for bone resorption under both normal and pathological conditions, such as osteoporosis [39]. The characteristic feature of an osteoclast is a ruffled edge where active resorption takes place [39]. Bone resorption by osteoclasts involves a series of steps such as pumping protons to solubilize the hydroxyapatite matrix, degradation of organic matrix by lysosomal enzyme cathepsin K, acid phosphatase, carbonic anhydrase II , all of which create an acidic environment (pH ~4.5) resulting in bone resorption [57-59]. The products of bone resorption can be measured in blood or urine and are discussed in section 1.3.4.

Osteocytes and mechanical sensing: Osteocytes are the most abundant and long-lived cells representing the terminal differentiation stage of osteoblasts. They regulate both remodelling and mineralization processes while being embedded in the mineralized bone matrix consisting of collagen and proteoglycans and glycoproteins and

gamma glutamic acid rich proteins [60]. Osteocytes are believed to play a role in skeletal homeostasis, mechanosensation and mechanotransduction (conversion of mechanical forces to biochemical signals) and as endocrine cells which can target organs such as muscle and kidney [61]. Data generated over the last few years have helped to identify molecules secreted by osteocytes such as sclerostin and fibroblast growth factor 23 (FGF-23) through which osteocytes control bone turnover. Osteocytes also express PTH/PTH-related peptide receptor PPR and have been shown to be involved in calcium homeostasis [62]. It was proposed back in 1970s, that osteocytes are involved in the early effects of PTH actions on the skeleton [63]. The rapid timing of hypercalcemic responses to PTH independent of an increase in number or activity of osteoclasts, the fact that rapid release of calcium from the bone does not require bone matrix breakdown and that calcium is released into the circulation at two different rates, Parfitt [63] concluded that osteocytes control the rapid release of calcium (from the less dense metabolically active bone around the osteocyte lacunae), whereas osteoclastic resorption determine the later phase of calcium released from bone in to the circulation. It has now been shown conclusively that PTH acts on osteocytes based on data from several in vitro and in vivo studies utilizing osteocytic cell lines, primary osteoblastic cell preparations and genetically modified mouse models [62, 64, 65]. This has also been confirmed in rodent models in which the PTH/PTHrP receptor (PPR) was deleted [64]. When subjected to a low calcium diet, hypocalcemia was evident in these animals [64] suggesting an important role of osteocytes in regulating calcium ion homeostasis. Based on these data, we hypothesize that suppression in PTH levels and bone resorption markers observed in acute calcium supplementation studies [50, 51, 66] may be an osteocytic response. This is of relevance to the current work and is discussed further in chapter 5.

1.3.2 Stages of Bone Remodelling

Bone remodelling comprises of six phases: namely quiescence, activation, resorption, reversal, formation and mineralization phase [46]. These occur at remodelling sites which could be randomly distributed and also targeted to the areas that require repair [44]. In the quiescent phase, the bone is at rest and the factors that

initiate the remodelling process are not completely understood [46]. Activation phase is characterized by recruitment, differentiation and fusion of the large multinucleated osteoclasts. These cells then attach to the mineralized bone surface and initiate the resorption phase by the secretion of hydrogen ions and lysosomal enzymes which can degrade all the components of bone matrix as explained above in section 1.3.1 [46, 67]. Osteoclast-mediated bone resorption takes only approximately 3-6 weeks during each remodelling cycle [47]. The reversal phase, as the name suggests, marks the transition of bone resorption to bone formation. Once osteoclasts have resorbed a part of the bone and created a resorption pit; they detach from the bone surface and are replaced by cells of the osteoblast lineage which in turn initiate bone formation. The coupling signals linking the end of bone resorption to the beginning of bone formation are as yet unknown but proposed coupling signal candidates include bone matrix-derived factors such as TGF-β, IGF-1, BMP, FGF-23 [46]. Osteoblasts synthesize extracellular matrix and begin to form osteoid which fills the resorption cavity or perforated areas [68] and is later mineralized to form bone. The mineralization process begins 30 days after deposition of the osteoid, ending at around 90 days in the trabecular and at around 130 days in the cortical bone [46]. Some osteoblasts differentiate into lining cells while osteoblasts within the osteoid become osteocytes and formation of new bone is completed. The quiescent or “at rest” phase then begins again [46].

1.3.3 Regulation of Bone Turnover

Bone remodelling is a highly complex process that is regulated tightly by genetic [69], mechanical [43], nutritional [70-73], local [56] and systemic factors [46, 56]. Mechanical factors such as high physical activity or lack of active lifestyle influence bone remodelling in accordance to Wolff’s law which states that the form or function of a bone is followed by adaptive changes in its internal architecture and its external shape [43, 74]. A persistent load is sensed by osteocytes network which increases bone formation. Likewise, an increase in bone resorption is observed when the load is reduced resulting in the bone becoming weaker and less metabolically demanding as seen during extended periods of bed rest or as observed in astronauts returning from prolonged periods in micro-gravitational environments [74, 75]. Nutritional factors such

as intake of calcium also influence bone remodelling. The rate of bone turnover triples from the age of 50 to 65 years in women and a high dietary calcium (1000-1500 mg/d) and vitamin D intake can reduce the remodelling rate to approximately premenopausal levels [73]. There is evidence to suggest that three year supplementation with vitamin K2-7 (menaquinone) reduces age-related decline in BMD at the lumbar spine and femoral neck in healthy post-menopausal women (n = 244) [76]. A systematic review and meta-analysis of randomized controlled trials report a 28% reduction in fracture risk, which is clinically relevant and significant [77]. However, the translation of this data to clinical practice is limited due to the high variability in study designs, co-supplementation with drugs or vitamin D and calcium and duration of trials, hence, further research is required [77]. The local factors include autocrine and paracrine molecules such as growth factors like BMPs, IGF-1, TGF-β, FGF, epidermal growth factor (EGF) and cytokines such as IL-1, IL-6, TNFs and prostaglandins produced by the bone cells [56]. Systemic factors of bone remodelling include hormones such as vitamin D metabolites, insulin, intermittent PTH, androgen, estrogen, calcitonin and thyroid hormones [46]. The local and systemic factors that control bone remodelling are listed in table 1.2. The role of calcium intake in controlling bone remodelling is discussed later in section 1.6.3.

Alterations or abnormalities in bone remodelling results in skeletal disorders [56] such as osteopetrosis (diminished bone resorption), paget's disease, osteoporosis (increased bone resorption) and inflammatory bone loss (excessive bone resorption) [56]. Obesity and diabetes mellitus (type 1 and 2) are two other metabolic conditions associated with low bone turnover that affect bone strength and increase fracture risk without reduction in BMD [55, 78, 79].

Table 1. 2 Hormonal and systemic regulators of bone remodelling

	Stimulates bone formation	**Decreases bone formation**	**Stimulates bone resorption**	**Inhibit bone resorption**
Hormones	Intermittent PTH [56, 80], Vitamin D metabolites, IGF-1 and IGF-2 [81], insulin [46], progesterone [46]	Glucocorticoids [46]	Glucocorticoids [82], thyroid hormones (hyperthyroidism) [46], high dose of vitamin D	Calcitonin [46], estrogens [83]
Growth factors	BMP-2, BMP-4, BMP-6, BMP-7[84],TGF-β [85], FGF [56]		TNFs, EGF, macrophage-colony stimulating factor (M-CSF), and granulocyte-macrophage colony stimulating factor (G-CSF) [56]	
Cytokines	IL-4, IL-13, osteoprotegerin (OPG) [56]		IL-1, IL-6, IL-8, IL-11 [56]	IL-4, interferon-gamma [56]

1.3.4 Clinical Assessment of Bone Turnover

Bone remodelling or turnover is assessed in clinical practice by the measurement of enzymes and proteins produced during bone formation and resorption processes. Collectively these plasma, serum or urine measures are known as Bone Turnover Markers (BTMs). BTMs mainly represent products of type I collagen with substantial post-translational modifications. Some BTMs are products of bone cell reflecting the number of particular cells within the bone environment [86]. A list of all the BTMs is given in table 1.3. These markers provide a qualitative and a quantitative assessment of the rate of bone turnover at any point in time, but their levels can be affected by a variation in physiological state as explained previously in table 1.1. Thus, appropriate care must be taken during the measurements of these BTMs to prevent preanalytical variability. The International Osteoporosis Foundation (IOF)-International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) Joint Working Group on Bone Marker Standards (IOF-IFCC WG-BMS) recommends the use of serum procollagen type I N propeptide (PINP) as the reference bone formation marker and plasma C-

terminal telopeptide of type I collagen (CTX-1) as the reference bone resorption marker to assess changes in bone metabolism in clinical studies [87]. The recommendation was based on clinical evidence from 22 prospective, cohort studies in which the association between incident fractures and BTMs was investigated [87]. Eighteen of these studies showed that one or more markers (such as OC, PICP, bone-ALP, CTX-1, NTX, DPD, TRACP etc.) were associated with risk of subsequent fracture. Further, the levels of bone resorption markers showed a more consistent association with fracture risk as compared to bone formation markers in both men and women [87]. In a meta-analysis of six prospective, cohort studies a moderate but significant association between baseline serum CTX-1 and PINP and the risk of future fractures was reported [88]. A one standard deviation increase in serum CTX-1 or PINP levels were associated with approximately 20% higher risk of fracture [88].

1.3.5 Clinical Utility of BTMs

The use of BTMs in clinical practice have several advantages over BMD measurements. These include i) monitoring early response to treatment ii) monitoring patient's compliance iii) prediction of bone loss iv) predicting fracture risk v) Identifying non-responders vi) selection of patients for antiresorptive therapy vii) selection of best choice of dose and dose frequency as a tool to modulate the treatment in individuals who do not show the expected response [86-91]. Furthermore, the measurement of BTMs has proved useful in drug development to establish proof of principle and to identify mechanism of action of drugs [87]. The decrease in BTM levels, particularly indices of bone resorption, occurs within hours with calcium supplementation, and days or weeks of starting treatment with antiresorptive therapy [87], such as with bisphosphonates. On the other hand, significant changes in BMD may take up to 18 months and hence treatment failures using DXA imaging are difficult to determine and the increment in BMD change is often modest [87]. Biochemical markers provide a superior signal-to-noise ratio (size of the change compared with the variability in the measurement) and therefore these measures may be used to shorten the duration of trials and to decrease study cohort size. For example, in a study of treatment of postmenopausal osteoporosis with alendronate, it was reported that the signal to noise

ratio for the bone turnover marker NTX was 4.9 whereas the ratio for lumbar spine BMD was only 1.3 after 6 months of treatment [92]. The bone markers measured in this thesis include bone ALP, osteocalcin and CTX-1. These are briefly discussed in the subsequent subsections.

Table 1. 3 List of BTMs

Markers	Abbreviation	Measured in
Resorption		
Amino-terminal cross linking telopeptide of type I collagen	NTX-1	Urine or serum
Carboxy-terminal cross-linking telopeptide of type I collagen	CTX-1	Serum/plasma (plasma preferred)
Deoxypyridinoline	DPD	Urine
Pyridinoline	PYD	Urine
Tartrate-resistant acid phosphatase	TRACP/TRAP5b	Serum
Formation		
Osteocalcin	OC	Serum or urine
Alkaline phosphatase (total)	ALP	Serum
Bone-specific alkaline phosphatase	BALP/Bone ALP	Serum
Procollagen type I C propeptide	P1CP	Serum
Procollagen type I N propeptide	PINP	Serum

Source [87]

1.3.5.1 Bone-Specific Alkaline phosphatase (Bone ALP)

The total serum alkaline phosphatase (ALP) consists of numerous dimeric isoforms originating from liver, bone, intestine, spleen, kidney and placenta. In healthy adults about 50% of the total ALP in the serum is of hepatic origin and the remaining is of bone origin [93]. Liver and bone ALP isoforms can be differentiated based on their physical properties (heat denaturation, electrophoresis, precipitation) or chemical properties (selective inhibition and immunoassay). Bone ALP is synthesized by the osteoblasts and is assumed to be involved in the calcification of bone matrix [94]. Bone ALP is a highly specific marker of osteoblast activity and is responsible for maturation and mineralization of newly formed osteoid. The levels of bone ALP can be measured in serum [95]. The Os Des Femmes De Lyon (OFELY) study showed that bone ALP levels were significantly associated with fracture risk in postmenopausal women (n = 435, 50-

89 years) independent of BMD [90]. Women with bone ALP levels in the highest quartile had significantly greater fracture risk as compared to women in the lower quartiles (relative risk 2.4, 95% CI 1.3-4.2). Osteocalcin and CTX-1 levels were also associated with higher fracture risk; however, results did not reach statistical significance. This could be due to differences between the predictive value of different markers [90]. Further, it has been reported that increased concentrations of bone formation markers are associated with a higher rate of bone loss in postmenopausal women [96, 97], hence could be equally predictive of higher fracture risk.

1.3.5.2 Osteocalcin

Osteocalcin is a highly specific osteoblastic marker which has been used for assessing relative degree of bone turnover in many clinical studies [56]. It contains 3 gamma carboxy glutamic acid (Gla) residues which confers calcium binding properties. Serum osteocalcin levels are used as a marker of osteoblastic activity [89, 94]. Some authors propose that osteocalcin may not be considered as a true bone formation marker as it is also liberated from the bone matrix during the bone resorption process [95]. Although there is evidence that lower circulating levels of osteocalcin are associated with an increased risk in all types of fractures in retrospective trials [98, 99], this relationship was not found to be significant in prospective trials [97, 98, 100, 101]. There is evidence to suggest that osteocalcin may play a role in regulating energy metabolism through insulin [102]. These data have clinical implications linked with the findings of the clinical study undertaken in this thesis and is discussed later in chapter 6, section 6.2.3.

1.3.5.3 Carboxy-Terminal Cross-Linking Telopeptide of Type 1 Collagen (CTX-1)

Type I collagen, a helical protein, is crosslinked at the N- and C-terminal ends of the molecule. During bone resorption, osteoclasts secrete a mixture of acid and enzymes (discussed in section 1.3) and degrade the collagen fibrils into molecular fragments. One of the fragments is C-terminal telopeptide. Post-menopausal women treated with antiresorptive therapy show a pronounced decrease in serum CTX-1 levels (around 50-80%), indicating that assay provides a clinically useful index of bone resorption [87]. CTX-1 has been recommended as reference bone resorption marker by IOF-IFCC WG-BMS

based on its specificity, well documented analytical and biological variability, availability of automated assay for serum and plasma, although plasma is a preferred specimen for CTX-1 measurements [87]. Foods or supplements containing calcium result in acute [50, 51, 66, 103, 104] and long-term suppression of CTX-1 levels [52, 105-108].

1.4 States of High and Low Bone Turnover- Osteoporosis and Obesity

Osteoporosis is a high bone turnover state associated with microarchitectural deterioration, reduced bone strength and higher fracture risk [109]. The effect of osteoporosis on bone health and role of nutrition in preventing osteoporosis is discussed in detail in the next section (section 1.5). The effects of low bone turnover in bone strength have not been established clearly. Emerging evidence suggest that conditions such as obesity and diabetes are associated with low bone turnover [55, 78, 110, 111]. Although individuals with higher body mass index (BMI) or people with type 2 diabetes present with higher BMD due to reduced bone turnover, the risk of fracture is not low in this population group [78, 79, 112]. The impact of obesity on bone health and role of nutritional factors to prevent fractures in this population group is discussed in section 1.10.

Obesity and osteoporosis share several features such as a common progenitor cell (MSC), genetic predisposition and both conditions are associated with adverse bone outcomes [113-115]. Further, both obesity and osteoporosis are amenable to prevention with lifestyle factors such as nutrition to some extent. While age related bone loss is inevitable, consuming adequate calcium and vitamin D can reduce the rate of bone breakdown, hence may delay the onset of osteoporosis [4, 15, 116-122]. This thesis aims to investigate the mechanisms of vitamin D to improve bone structure in a cohort of elderly men and women and the role of calcium in improving bone outcomes in obese postmenopausal women.

1.5 Pathogenesis of Osteoporosis

Osteoporosis is defined as a skeletal disorder characterized by enhanced skeletal fragility due to reduction in both bone quantity (low BMD) and bone quality

(microarchitectural deterioration) [123] (figure 1.1). Osteoporosis is the most prevalent metabolic bone disease across the globe and effects over 1 million Australians . Osteoporosis imposes significant morbidity, premature mortality and is a major cost burden to the society [124]. About 4.74 million Australians over 50 years of age have osteoporosis or osteopenia and the number is projected to rise to 6.2 million by the year 2022 [124]. Out of 4.74 million people, 22% have osteoporosis and 78% suffer from osteopenia, a condition which precedes osteoporosis. There is 1 hip fracture every 3.6 minutes in Australia and by 2022 there will be 1 hip fracture every 2.9 minutes. In 2012, the cost to treat osteoporotic fractures was $2.75 Bn and is estimated to rise to $33.6Bn over the next 10 years [124]. In Australia, there were 85,509 hospitalizations for low trauma fractures in 2016-17 [125]. Globally, osteoporosis account for more than 8.9 million fractures annually, resulting in an osteoporotic fracture every 3 seconds [126]. Despite this, osteoporosis remains considerably underdiagnosed and undertreated and as many as 80% of the patients at high risk are neither identified nor treated [127, 128]. Therefore, the need for preventive nutritional strategies are important to prevent primary and secondary fractures in at-risk patients.

Osteoporosis is not a disease that is restricted to women with men accounting for up to 30% of all low trauma fractures and their associated costs [124]. Further, men tend to have worse outcomes after fracture than women and are twice as likely to die after hip fracture as compared to women [129]

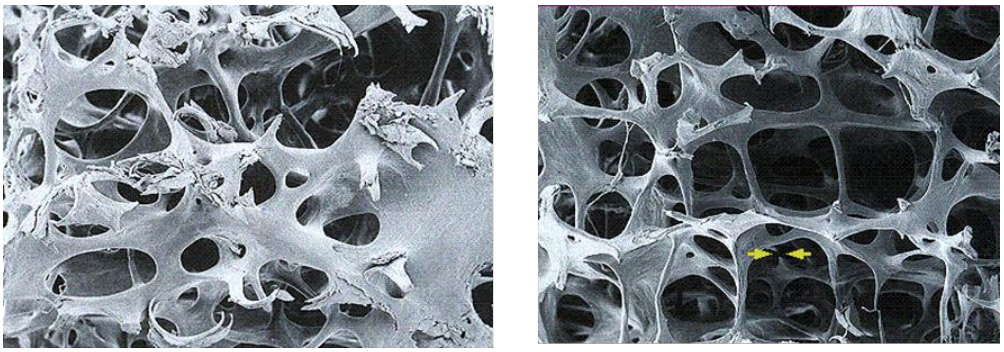

Figure 1. 1 Representative image of healthy and osteoporotic bone

1.5.1 Aetiology

Osteoporosis is a heterogenous disorder with a multifactorial etiology. Some of the risk factors are non-modifiable such as female sex, menopausal age, use of certain

medications such as glucocorticoids, other metabolic disorders and ethnicity [4, 130, 131]. However, there are several readily modifiable risk factors that can be changed to reduce the risk of osteoporosis. These include inadequate dietary calcium intake and vitamin D deficiency [4, 12, 73, 121, 132-137], low or high body mass index (BMI) [112, 138-140], lack of weight-bearing exercise [141], fall prevention [4], excessive alcohol consumption and smoking [4, 130]. This review is restricted to role of nutritional factors, age and estrogen deficiency in the causation of osteoporosis. However, the multiple etiologies of osteoporosis are acknowledged but a discussion of all the factors is beyond the scope of this review.

1.5.1.1 Estrogen Deficiency

Estrogen deficiency has been shown to cause a 10% reduction in spine and hip BMD and an 8% reduction in total body calcium during a five-year interval around menopause [142]. Estrogen deficiency results in bone loss directly through action on bone cells and indirectly through the loss of action of estrogen on the intestine and kidney [143]. During pre-menopausal years, when estrogen levels are adequate, it suppresses the activation of BMUs and maintains a balance between bone resorption and formation. However, around menopause, when estrogen levels start to decline, the activation frequency of new BMUs increases with bone resorption rate exceeding bone formation [130, 144]. Cellular changes associated with estrogen deficiency include increased osteoblastogenesis, increased osteoclastogenesis, increased lifespan of osteoclast and reduced lifespan of osteoblasts and osteocytes [145]. Estrogen deficiency is associated with increased production of TNF-α, IL-1, which causes stimulation of stromal cells and pre-osteoblasts to release several factors including IL-6, IL-11, M-CSF, GM-CSF, TGFβ and RANKL. All these factors stimulate osteoclastogenesis. Osteoclastogenesis is promoted by the binding of RANKL to its receptor, nuclear factor kappa-B (RANK). Estrogen deficiency also causes a reduction in osteoprotegerin (OPG) levels, a soluble protein receptor for RANKL and prevents RANKL mediated activation and differentiation of osteoclasts. Reduction in OPG levels results in higher RANKL being available to bind to RANK thus stimulating osteoclast differentiation and activity. Further, higher levels of M-CSF, IL-1, TNF, IL-6 and RANKL prevent osteoclast apoptosis and enhances osteoblasts apoptosis [82, 130].

There is evidence to suggest that estrogen levels positively affect calcium metabolism and its deficiency disrupts calcium economy at menopause without any change in PTH levels [146]. In a longitudinal study assessing bone-related biochemical changes at menopause in 104 premenopausal women, Nordin et al., demonstrated that menopause (after 8 years follow-up) is associated with a rise in serum ionized calcium without any change in PTH levels, suggesting a change in the ionized calcium PTH set-point [147]. Furthermore, a reduction in intestinal calcium absorption without any change in serum 1,25-dihydroxyvitamin D_3 ($1,25(OH)_2D$) levels accompanied by a decrease in renal tubular reabsorption of calcium reflects the loss of an estrogen dependent action on these organ systems [147]. Increased bone resorption markers despite normal PTH levels suggests an increased sensitivity of bone to the action of PTH [147, 148]. Estrogen administration has been shown to reverse these effects by lowering urinary calcium excretion, reducing urinary excretion of products of bone collagen metabolism (hydroxyproline) and reduction in serum alkaline phosphatase activity [146]. The discovery of estrogen receptors in the kidney [149] gave rise to the hypothesis that estrogen may play a direct role in calcium reabsorption in the renal tubules. The reduction in calcium absorption is reversible with replacement of estrogen suggesting a direct role of estrogen in intestinal calcium absorption [150]. Calcium absorption is regarded as the main determinant of calcium balance, accounting for nearly 60% of the variance in calcium balance [17]. The estrogen deficiency caused by menopause leads to a negative calcium balance and affects the calcium economy increasing the risk of osteoporosis. Furthermore, the rate of calcium absorption mainly via active transport, when adjusted for age, was shown to be significantly reduced in postmenopausal women with prevalent fractures [151]. Thus, increasing calcium intake at this stage has a therapeutic effect which can offset some of these biochemical changes and prevent bone loss [15, 152].

1.5.1.2 Age

While estrogen deficiency plays a central role in the pathogenesis of osteoporosis both in women and men, epidemiological evidence suggests that age is also associated with an early loss of trabecular bone mass in both sexes (much before any changes in sex-hormone levels are observed) [153], secondary hyperparathyroidism

[154-156] and increased generation of reactive oxygen species (ROS) with aging [157]. Based on the changes in volumetric BMD observed in two population based longitudinal studies, Riggs et al., [158, 159] reported three phases of bone loss in women: 1) an early phase of trabecular bone loss in late premenopausal women (2) a transient phase of rapid cortical bone loss at menopause and an acceleration of pre-existing trabecular bone loss, and (3) a subsequent slower phase of loss involving similar amounts of cortical and trabecular bone that continues through the later years [158, 159].

Early phase of trabecular bone loss: Epidemiological studies have shown that bone loss begins soon after achievement of peak bone mass in young women between the ages 21 and 50 years (or until menopause) which accounts for about 30% of their total trabecular bone loss over life [153, 158]. No association between this trabecular bone loss and sex hormone levels were found [158]. In contrast, loss in cortical bone mass has not been observed in women until about 5 years before menopause [160]. Adult men (ages 20–50 years) also experience an early phase of trabecular bone loss similar to premenopausal women with no significant changes in cortical bone mass [158]. Though the mechanism for this early sex hormone independent bone loss in young men and women is not completely understood, it challenges the prevailing view that estrogen deficiency explains most of the age-related bone loss. Kveiborg and colleagues proposed that age-related decrease in skeletal IGF-1 [161] could drive this early loss of trabecular bone loss. Khosla et al., [162] showed that higher levels of estrogen are required to prevent trabecular bone loss compared with cortical bone loss which could partially explain why cortical bone loss is not observed until changes in sex hormone levels occur.

Accelerated transient phase of postmenopausal bone moss: Menopause results in a rapid phase of cortical bone loss and acceleration in the pre-existing phase of trabecular bone loss lasting for about 4–8 years [144]. This has already been discussed above in section 1.5.1.1.

The late, slow phase of bone loss: The slow late phase involves progressive and continuous loss of bone mass both in men and women at the rate of about 0.5%-1% per year in men and 1-2% in women [144] and primarily involves the loss of cortical bone

[163]. The main causes of this slow progressive loss of bone mass in elderly women include secondary hyperparathyroidism caused by long term estrogen deficiency resulting in elevated PTH levels and corresponding increase in bone resorption [156]. The serum PTH levels begins to increase in women about 10-15 years after menopause [144]. A 24-hour calcium infusion study to suppress PTH levels in elderly postmenopausal women (n = 10; at least 12 years post menopause) showed that elevated levels of BTMs observed on the control day were no longer present on the calcium infusion day, suggesting that the increased serum PTH was possibly the cause of the increased bone turnover [154]. In another study, elderly women who were maintained for 3 year on high calcium intake (approx. 2400 mg/day) had significantly lower PTH levels ($p < 0.001$) and reduced parathyroid gland secretary capacity ($p < 0.005$) as compared to women maintained at calcium intakes of around 800 mg/day. A reference group of young women with calcium intake of approximately 900 mg/day was also included in the study. The elderly women with calcium intake of 800 mg/day had 70% higher PTH levels as compared to the reference group while the PTH levels were similar in the high calcium group and the reference group [121]. These data indicate that failure of elderly women to increase their calcium intake to offset age-related increases in calcium requirement may contribute to development of increased parathyroid activity.

In fact, several studies have shown that combined calcium and vitamin D supplementation in elderly women can reduce bone loss and fracture risk [2, 20, 25, 143, 164, 165]. Serum PTH levels continues to increase throughout the later years which could be partly due to abnormal parathyroid gland function [166, 167]. Further, vitamin D deficiency associated with aging independently contributes to secondary hyperparathyroidism [156]. Taken together, these data highlight the importance of consuming adequate calcium intake and vitamin D supplementation to reduce age-related loss of bone mass in the elderly population.

Decreased bone formation has also been implicated in postmenopausal bone loss [168]. Histological evidence demonstrate that late postmenopausal women have decreased trabecular wall thickness indicating reduced bone formation at the tissue level [168]. This is further supported by the fact that at menopause, bone resorption

markers increase by 79-97% while the increase in bone formation markers is only by 37-52% [91]. Studies in rodent models have shown that aging is associated with increased levels of oxidative stress markers in osteoblastic cells which may result in impaired bone formation with aging [157, 169-171].

1.5.1.3 Nutritional Factors

Nutrition plays a central role in the pathogenesis, prevention and treatment of osteoporosis [2, 16, 122, 134, 137, 152]. Calcium homeostasis is the major nutritional factor in maintaining the skeleton; however numerous other nutrients that contribute to bone health have been identified [1]. Food and Nutrition Board of the Institutes of Medicine have designated calcium, phosphorus, vitamin D and magnesium as bone related nutrients [172]. Other nutrients implicated in bone health are vitamin K, especially vitamin K2-7 or menaquinone, vitamin C, and potassium [137]. Beneficial dietary patterns include higher protein intake especially dairy protein, high intake of fruit and vegetable and an alkaline diet [173, 174]. The role of calcium and vitamin D is of interest for this thesis and is discussed later in section 1.6 and 1.7, respectively.

1.5.2 Assessment of Osteoporosis

As defined above, osteoporosis is skeletal disorder characterized by changes in both bone quantity and quality resulting in reduced bone strength. Changes in bone quantity are reflected by variations in BMD measures. The assessment of bone quality includes bone microarchitecture, bone turnover, accumulation of micro-fracture and degree of bone mineralization. The contribution of each of these factors in determining fractures risk and their assessment is discussed below.

1.5.2.1 BMD and Fracture risk

In clinical settings, BMD is measured using dual-energy X-ray absorptiometry (DXA) which is the gold standard method used for diagnosing, prognosis and selection of cases for osteoporotic treatment to lower fracture risk [143, 175]. DXA imaging models the bone as a two-dimensional object (2D) and measures areal BMD (aBMD) rather than volumetric bone mineral density (vBMD) which is a 3D measure. DXA does

not differentiate between the measurements of trabecular and cortical bone compartments. BMD is expressed by its relationship to two norms: the expected BMD for young healthy adults of the same sex (standard deviations from the young normal mean; T score); and the expected BMD for the patient's age and sex (Z score). The difference between the patient's score and the norm is expressed in terms of standard deviation (SD) above or below the mean. In general, a one-standard deviation reduction represents a loss of 10–12% BMD [176]. The World Health Organization (WHO) established four categories relating to BMD measurements in postmenopausal women which are regarded as diagnostic criterions for osteoporosis [177]. Normal category includes women with T-score at or above -1 SD, osteopenia is T-score between -1 and -2.5 SD, osteoporosis is when T score is at or below -2.5 SD and severe osteoporosis includes women with T score below -2.5 and previous fragility fracture [177].

Skeletal assessment using DXA is typically done in the lumbar vertebrae and total hip. However, the DXA measurement of the vertebrae is often affected by degenerative artefacts in the spine. Therefore, total hip measurement provides the reference standard for the diagnosis of osteoporosis and is used for fracture risk assessment [178, 179]. The principal difficulty for assessment of fracture risk based on WHO definition is that this threshold value has high specificity but low sensitivity. Studies have shown that patients who experience a low trauma fracture and those who do not, have a substantial overlap in BMD values [180, 181]. Furthermore, the proportion of women with osteopenia is about six-fold that of osteoporosis, therefore the number of fractures will be greater in those with osteopenia [182].

To overcome these problems, web-based fracture risk calculators have been developed which takes well known risk factors (discussed in section 1.5.1) and estimates the absolute risk of osteoporotic and hip fracture within a 5 and/or 10-year period for an individual. The WHO sponsored tool, called Fracture risk assessment tool (FRAX), is a country-specific algorithm developed by University of Sheffield, UK which calculates 10-year fracture risk based on ten clinical risk factors with or without BMD and was developed using data from 20 population based studies [183, 184]. The clinical risk factors included in the FRAX tool are age, BMI, sex, previous fracture, parent fracture history, current smoking, glucocorticoid use, rheumatoid arthritis, secondary

osteoporosis, alcohol 3 or more units per day. Studies have shown that incorporation of FRAX improved fracture prediction [184]. The Garvan Bone Fracture Risk Calculator was developed by the Garvan Institute of Medical Research (Sydney) using data from the Dubbo osteoporosis epidemiology study. This calculator estimates 5- and 10-year risk of fracture, based on four clinical risk factors which include age, sex, number of fractures and number of falls and BMD [185]. A nomogram published in 2007 using femoral neck BMD T score with other clinical risk factors to predict the 5 and 10 year hip fracture risk in men and women in the primary care setting showed that incorporation of clinical risk factors enhanced the performance of BMD in the prediction of osteoporotic risk in both men and women [185]. However, several studies suggest that both these FRAX tools still lack sensitivity, specificity and positive predictive value for osteoporotic fracture [186] and still fail to identify many who go on to sustain a fracture [187]. In the Newcastle Thousand Families Study (NTFS) cohort, femoral neck BMD or lumbar spine BMD or FRAX scores did not differ between participants with and without vertebral fractures [188]. These observations raise an important point that some other factors besides BMD and established clinical risk factors influence fracture risk. Over the last two decades, advancement in imaging technologies have enabled the assessment of bone quality in both trabecular and cortical compartments that influence fracture risk independent of BMD values [41, 42, 189-195].

1.5.2.2 Bone Quality – Components and Fracture Risk

Bone quality comprises of four physiological and structural indices: i) rate of bone turnover ii) bone architecture and geometry, iii) properties of the collagen-mineral matrix and iv) microdamage accumulation [38]. These components are largely interdependent such that an abnormality in one of these parameters may result in changes in others. Bone turnover is a major determinant of all the other components of bone quality and hence its measurement in clinical practice is important [86] and has already been discussed in section 1.3.4. The assessment of bone microarchitecture is discussed below. The measurement of the extent or degree of bone mineralization, micro damage accumulation and bone matrix and mineral composition are also important determinants of bone quality [109] but a discussion of all these variables is beyond the scope for this review.

1.5.2.2.1 Bone Microarchitecture- Definition and Assessment

The importance of bone microarchitecture has been highlighted in several large clinical studies which suggests that deficits in trabecular and cortical bone structure are significant contributors of fracture risk independent of BMD or FRAX scores in both men and women [41, 42, 189-195]. Bone microarchitecture can be non-invasively assessed using micro-computed tomography (μCT) with high resolution and is successfully used in research settings employing bone biopsies obtained from humans or animal models [196, 197]. Clinical CT systems can be used for non-invasive imaging, but the resolution is insufficient to measure bone microarchitecture [198]. High resolution peripheral quantitative computed tomography (HR-pQCT) is one of the best and validated research tool to measure bone microarchitecture and changes in cortical and trabecular bone compartments associated with aging and osteoporosis [41, 42, 189-195, 199]. In addition, HR-pQCT has the potential to yield biomechanical information using finite element analysis (FEA). A limitation of HR-pQCT is that measurements are confined to peripheral sites such as the distal radius and distal tibia. Magnetic resonance imaging (MRI) measures bone microarchitecture without ionizing radiation, however, it is a very expensive technique and requires long scan times to generate high resolution images of trabecular bone [200, 201]. The trabecular bone score (TBS) is another novel software application that assess bone texture using lumbar spine BMD scans. An integrated software analyses pixel gray-level and spatial variation in the DXA scan providing an indirect index of bone microarchitecture [202].

In research settings, μCT is often used to assess bone microarchitecture using representative bone biopsy specimens obtained from elderly patients [109, 203-207]. The variables used to define trabecular bone microarchitecture as per ASBMR classification are explained in table 1.4. Briefly, the architecture of trabecular bone can be described by the number (Tb.N), thickness (Tb.Th) or distance (Tb.Sp) between the trabeculae or the shape of trabeculae. In healthy bone, trabeculae tend to be shaped as plates rather than as circular or elliptical rods which provides greater strength in the load-bearing direction. The extent to which the trabeculae are plate-like or rod-like is called the Structure Model Index (SMI). Bone surface to bone volume ratio (BS/BV) is another variable that defines the texture and thickness of the bone. The trabecular bone

with more plate like configuration will have lower BS/BV and thus stronger as compared to bone with more rod-like configuration and higher BS/BV. When people begin to lose bone, as in osteoporosis, the plate like trabecular bone structure becomes more rod-like, which leads to increased BS/BV and reduced connectivity [38]. These alterations in trabecular bone structure may contribute to changes in bone quality and strength. Using iliac crest biopsies, Kleerekoper and colleagues showed that patients with vertebral fracture have lower Tb.N and connectivity as compared to the age and bone mass matched non fracture control group [208]. Likewise, several studies have reported significantly lower Tb.N, increased Tb.Sp and prevalence of rod-like trabecular structure in fractured neck of femur patients as compared to controls [203-207]. Further, studies have also shown that bone strength calculated from fracture toughness testing is negatively associated with BS/BV and SMI and positively with BV/TV and Tb/N suggesting that as the trabeculae become more rod-like in structure, less force is required to fracture the bone [206, 209].

Biomechanical studies demonstrate that the structural properties of bone specimens are also determined by cortical bone [210]. Cortical bone strength is dependent on its material and microarchitecture properties and are defined by parameters such as cortical porosity [211] and thickness [210]. Increased bone resorption on the endocortical surface and haversian canal lead to higher cortical porosity which is a significant determinant of cortical bone strength [211-214]. Cortical thickness has also been reported to be a significant predictor of fracture load at the distal radius [210]. Furthermore, thinning of the cortex occurs with aging [215] and disease [216] and has been shown to result in bone fragility and increased fracture risk [215, 217].

Vitamin D has been shown to be associated positively with bone health [19, 21, 22, 132, 218-230] and is known to reduce fracture risk when co-administered with calcium [18, 20, 165, 231-238] but there is very little evidence of reduction in bone loss or improvement in BMD with vitamin D supplementation alone in elderly people [36, 239-242]. We hypothesize that vitamin D may act to improve bone microarchitecture, hence in this thesis we have attempted to understand the relationship between vitamin D and trabecular microarchitecture. The role of vitamin D supplementation in improving

bone health remains an ongoing debate and will be discussed further in the section 1.8.

Table 1. 4 Variables commonly used in describing trabecular microarchitecture

Variable	Abbreviation	Units	Definition
Tissue volume	TV	mm^{-1}	TV index is determined by counting the voxels of the bone tissue within a given volume of interest (VOI).
Bone volume	BV	mm^{-1}	The percentage of area within the TV that emits the signal indicating accumulated mineral (BV).
Bone volume/tissue volume	BV/TV	%	BV/TV is the ratio of bone volume within the VOI and is represents the mineral content of the bone specimen.
Trabecular thickness	Tb.Th	mm	The thickness of the individual trabeculae. It is calculated using spheres whose diameter must fill the trabecular structure.
Bone surface/bone volume	BS/BV	mm^{-1}	The measurement of BS/BV provides an integrated measure of thickness and texture of the bone. BS/BV is inversely related to Tb.th i.e. a thicker bone with a higher number of trabecular plates will have a lower bone surface to bone volume ratio.
Trabecular separation	Tb.Sp	mm	Tb.Sp is measured as the average distance between trabecular surfaces. This typically increases with loss of trabecular bone
Trabecular number	Tb.N	mm^{-1}	The number of trabeculae in each bone volume
Structural model index	SMI		SMI indicates the relative prevalence of rods and plates in a 3D structure of trabecular bone. The osteoporotic degradation of trabecular bone which is characterized by a transition from plate-like to rod-like architecture, where a structure with only plates or rods will have values of SMI 0 and 3 respectively, showing higher values for the osteoporotic groups.
Trabecular pattern factor	Tb.Pf	mm^{-1}	Tb.Pf is an indicator of the surface curvature. A well-connected trabecular bone has concave curvature indicating higher connectivity with lower Tb.Pf values. Convex curvature is characteristic of disconnected structures which increases Tb.Pf

Source [243, 244]

1.6 Calcium and Bone Health

1.6.1 Calcium – Requirements and Functions

Calcium is essential component of bone mineral and thus, adequate calcium intake is considered important for skeletal health. Skeletal tissue acts as a reservoir for calcium which can be utilized through the process of bone resorption when dietary

calcium is inadequate to make-up for obligatory calcium losses and maintenance of plasma calcium levels. This mobilization of skeletal calcium results in bone mass reduction and will eventually lead to osteoporosis, if left untreated [245]. For this reason, Heaney described osteoporosis as the “index disease” of calcium deficiency [245] .

Adequate plasma calcium is required for several key functions like maintenance of cellular structure, metabolic function, signal transmission, muscle contractions activities of enzymes and normal clotting of blood [246]. Ionized calcium is the biologically active form of calcium that translates external signals into internal signals in the cells. Serum calcium levels are tightly regulated within very narrow range (2.25–2.5 mmol/L) [246].

The Institute of Medicine (IOM) recommends for ages 51 to 70 years, 1000mg/day for men and 1200 mg/day for women and 1200 mg/day for everyone aged 70 years or more [247]. Inadequate dietary intake of calcium across life stages has been reported worldwide [248]. The National Health and Medical Research Council for Australia and New Zealand defines nutrient reference values as recommended dietary intake (RDI) and estimated average requirement (EAR) [249]. RDI refers to the average daily dietary intake level that is enough to meet the nutrient requirements of nearly 97–98% healthy individuals in a life stage and sex. EAR is the median requirements for the respective age group and sex. RDI of calcium is set at 1300 mg for women over 50 years and men above 70 years. As per the latest Australian Institute for Health and Welfare (AIHW), mean intake of calcium among Australians is less than the EAR, around 91% of females and 63% of males in the age range 51-70 years had calcium intakes less than the EAR. Prevalence of inadequacy (proportion of people consuming calcium less than the EAR) is equally high among males and females above 71 years, the values being 90% and 94%, respectively [249, 250] . The EAR and mean intake for Australian population is summarized in table 1.5.

In older people, a diet containing lower levels of calcium may not compensate for the obligatory losses of calcium and increased calcium requirements as explained above and may predispose them to the risk of osteoporosis and may eventually result

in fragility fractures [2, 15, 16, 117, 120, 134, 143, 147, 152, 164, 251-253]. Hence, increasing calcium intakes through dietary sources or supplements (when it is not possible to increase dietary calcium intake) may significantly contribute to the prevention of bone loss and osteoporosis.

Table 1. 5 Comparison of mean calcium intakes among Australians with EAR [249, 250]

	Males		Females	
Age	**Mean intake (mg/day)**	**EAR (mg/day)**	**Mean Intake (mg/day)**	**EAR (mg/day)**
51-70	781	840	741	1100
>70 years	762	1100	690	1100

1.6.2 Absorption and Regulation of Calcium Levels

Calcium is absorbed in the intestine through a saturable active transcellular process and a non-saturable passive paracellular diffusion mechanism. The active transcellular process of calcium absorption is dependent on $1,25(OH)_2D$ levels, intestinal vitamin D receptor (VDR) and the up-regulation of the $1,25(OH)_2D$ responsive genes including the calcium transport protein called transient receptor potential vanniloid (TRPV) family, especially TRPV 6 and the calcium binding protein calbindin-D_{9k} [254, 255]. At low and moderate calcium intake, the active transport is the dominant mechanism for calcium absorption and occurs primarily in the duodenum where VDR is expressed in the highest concentration and to some extent in the jejunum. Calcium absorption through the active process is an ATP dependent pathway and is regulated by nutritional and physiological factors, with increased absorption at low dietary calcium intake [256]. As the calcium intake increases, the active component becomes saturated and vitamin D-mediated synthesis of calbindin-D_{9k} decreases [246, 254]. At higher calcium intake, most of the calcium absorption occurs through a non-saturable passive diffusion pathway which involves the movement of calcium between mucosal cells across the concentration gradient. It has been shown that the maximum diffusion of calcium occurs in the duodenum, jejunum, and ileum [256, 257].

Calcium intake, vitamin D levels, age, diet composition (diets containing interfering substances such as high fiber that reduces calcium bioavailability), ethnicity,

genetics, smoking (reduces calcium absorption) and estrogen are some of the factors that influence calcium absorption efficiency [258]. Calcium absorption efficiency is inversely related to calcium load, though the absolute quantity of calcium absorbed increases with increased load [259]. Fractional calcium absorption, which is the percentage of a given dose of calcium that is absorbed, varies inversely with calcium intake [260]. However, isotopic tracer techniques which can accurately measure calcium absorption demonstrate that the increase in fractional calcium absorption is not sufficient to offset the loss in absorbed calcium that occurs as a result of the lower intake of calcium and thus net calcium absorption is reduced [246]. For instance, a study showed that when calcium intake was lowered from 2,000 to 300 mg, in 30 healthy women, the fractional calcium absorption increased from 27% to about 37% [261].

Fractional calcium absorption has been reported to decline with aging and after menopause [147, 253]. Menopause is associated with an explicit and sustained rise in excreted calcium. Since calcium absorption falls slightly at menopause, this extra excreted calcium is considered as an increase in obligatory calcium loss and may partly explain the rise in calcium requirement in post-menopausal women [134].

Maintaining the levels of circulating ionized calcium within a narrow physiological range is critical for the body to function normally and is tightly regulated by a tri-axial system comprising of bone, kidney and intestine through the action of three main hormones namely $1,25(OH)_2D$, PTH and calcitonin. These organs and hormones work together to maintain plasma calcium levels within this narrow range [246, 254].

PTH is central to the operation of this tri-axial system. When plasma calcium levels fall, due to inadequate dietary intake or other causes, it is sensed by the calcium sensing receptors (CaSR) present on the PTH gland which results in increased production and release of PTH into the circulation [254]. Once released, PTH along with $1,25(OH)_2D$ acts on the bone and mediates osteoblast-induced differentiation of osteoclasts through the RANK–RANKL signaling pathways. Upon stimulation of osteoblasts, the cells increase their production of the pro-osteoclastic factor RANKL and reduces the expression of the anti-osteoclastic factor OPG. Downregulation of OPG permits the binding of RANKL to RANK resulting in osteoclast mediated bone resorption and release of calcium from the

bone into the blood stream [254, 255]. Further, PTH can stimulate the secretion of M-CSF, another factor that promotes osteoclast differentiation [262]. PTH can also acts via the osteocytes to maintain calcium homeostasis as explained previously in section 1.3.1.

At the kidney, PTH stimulates calcium reabsorption, suppresses renal phosphate reabsorption and finally stimulates renal enzyme 25-hydroxyvitamin D3 1α-hydroxylase (CYP27B1), that catalyzes the conversion of 25 hydroxy vitamin D (25(OH)D) to $1,25(OH)_2D$, the hormonally active form of vitamin D, that stimulates bone resorption, renal calcium reabsorption in the distal convoluted tubule, and active calcium absorption in the proximal small intestine [246]. Together, $1,25(OH)_2D$ and PTH levels serve to increase serum calcium levels at the expense of bone. The cumulative effect of these actions is to maintain plasma calcium levels. It is essential to state that these three effects are independent of one another and serum PTH levels are inversely related to the levels of calcium made available by the combined effect of all three mechanisms [246].

Apart from PTH and $1,25(OH)_2D$, few other hormones or physiological states have been shown to exert direct or indirect effect on calcium absorption. Prolactin can produce direct and indirect effects to increase intestinal calcium absorption during pregnancy and lactation. Studies have shown that treatment with glucocorticoid reduces calcium absorption by 31% without any significant reduction in $1,25(OH)_2D$ levels. Earlier studies by our group have demonstrated the existence of glucocorticoid-induced intestinal resistance to vitamin D action [263]. FGF-23, growth hormones (GH) and thyroid hormones may influence calcium absorption by influencing renal $1,25(OH)_2D$ production, though this need to be tested in further studies [254].

1.6.3 Calcium and BTMs

Increases in calcium intake, within the range 1000-1300 mg induces acute [50, 51, 66, 116, 264] and long-term suppression [52, 108, 265-271] in PTH and CTX-1 levels in post-menopausal women and men. The extent of reduction is dependent on the calcium dose and source [52] and the vitamin D status of the population [265]. The reduction is higher in the elderly, possibly because older people generally have higher

remodelling rates than younger people with comparable calcium intakes. In a 4-year randomized, double-blind, placebo-controlled trial (RCT) in 236 postmenopausal women, consuming 1600 mg calcium resulted in 18.9% ($p = 0.002$) reduction in PTH levels, 11.9% ($p = 0.026$) reduction in serum OC and 32.2% ($p = 0.003$) reduction in PYD with a 0.9% increase in total bone density at 4 years ($p = 0.017$)[270].

1.6.4 Calcium and BMD

Many well-designed RCTs examining the effect of calcium on changes in BMD have been conducted. Nordin [15] summarized the effect of calcium supplementation in 32 controlled trials (n = 3169) in postmenopausal women and reported that 1000 mg calcium/day had a significant preventive effect on bone loss of 0.8% over an average of 2 years, with the effects lost after 4 years of stopping the calcium supplementation. Supplements of less than 700 mg were not effective, but higher doses had no additional benefit on preventing bone loss [15]. A summary of 37 controlled calcium intervention trials showed that increasing calcium intake reduced bone loss or osteoporotic fractures at one or more skeletal site, except in two studies [3]. The trials which failed to show an impact of calcium supplementation included people with sufficient baseline intake or early menopausal where bone loss is mainly related to excessive bone resorption caused by estrogen deficiency which limits the ability to measure the efficacy of calcium [3]. Another meta-analysis of 12 trials conducted in postmenopausal women showed that 1000mg calcium supplementation reduced the rate of bone loss by an average of 0.8%-1% per year at all sites except the vertebrae [272]. A further meta-analysis of 15 trials (n = 1806) reported an average reduction in bone loss by 2% in total body BMD, 1.7% at the lumbar spine, 1.6% at the hip and 2% at the distal radius after two or more years of calcium supplementation as compared to placebo [273]. Another meta-analyses undertaken to understand the role of dietary calcium versus calcium supplements on BMD demonstrated that increasing calcium intake from dietary sources increased the total body BMD and total hip BMD by 0.6-1.0% after one year and by 0.7-1.8% at the lumbar spine and femoral neck after two years of supplementation. Calcium supplements increased BMD by 0.7-1.8% at all these skeletal sites at one, two, and over two and a half years [274]. Reid et al., reported a 1-1.5% increase in spine and hip BMD

in healthy men (age ≥40 years) supplemented with 1200 mg calcium/day for two years compared to the control group but no effect on BMD was observed when calcium dose was 600 mg/day [275]. Further, studies have shown that the effects of calcium supplementation are lost within two years of stopping the supplements suggesting that adequate intake of these nutrients must be maintained to retain the skeletal benefit [276].

1.6.5 Calcium and Fracture Risk

Several researchers have described the positive effects of calcium supplementation on fracture prevention [270, 277-280]. A 72% (HR: 0.28; 95% CI: 0.09,0.85) reduction in risk of overall fracture was reported in an RCT involving 930 men and women receiving 1200mg of calcium over 4 years as compared to placebo group. There were 15 minimal trauma fractures out of 464 participants in the calcium group as compared to 29 low trauma fractures out of 466 participants in the placebo group [279]. However the HR reverted to 1.10 (95% CI: 0.71, 1.69) during the post treatment 10 year follow up when the subjects were informed about the type of treatment they received and had the choice to take or not to take calcium [279]. These data again highlight the importance of sustained and regular intake to maintain the benefit of calcium on skeletal health. In another RCT, calcium supplementation at 1200mg/day in 197 postmenopausal women with (n = 94) or without (n = 103) pre-existing spine fractures resulted in significant reduction in the rate of vertebral fracture in high-risk women with pre-existing fractures. A total of 15 women out of 53 on calcium had fractures as opposed to 21 women out of 41 in the control group (p = 0.023). No difference in risk reduction was found in the women without pre-existing fractures [278]

In a meta-analysis of 14 trials of calcium supplementation and fracture risk, a 4% (odds ratio 0.96, 95% CI 0.93-0.99) reduction in fracture risk was reported for each 300 mg increase in calcium intake [281]. Another meta-analyses reported a 23% reduction in vertebral fracture (RR 0.77; 95% CI 0.54–1.09) and a 14% (RR 0.86; 95% CI 0.43–1.72) reduction in risk of non-vertebral fractures [273]. A meta-analysis by Tang et al., of 17 randomized controlled trials (n = 52,625) reported a 12% (RR 0.88, 95% CI 0.83-0.95, p = 0.0004) reduction in risk of all types of fractures with calcium and calcium in

combination with vitamin D [236].

Overall, the current evidence points to beneficial effect of calcium in lowering bone remodelling markers, modest increases BMD and reduction in the risk of fracture in both men and women.

1.6.6 Toxic Effects of Calcium Supplementation

There has been a considerable debate over use of calcium supplementation and risk of developing kidney stones [282, 283] and myocardial infarction [284, 285]. Heaney et al. reported no increase in formation of kidney stone with high calcium intake obtained from diet or supplements. On the contrary, these authors found an inverse relationship between calcium intake and stone risk [286]. In a further meta-analyses of studies including only post-menopausal or elderly women and incidence of kidney stone reported no effect of calcium supplementation as compared to placebo [287]. Further, secondary analyses of a study including 5452 postmenopausal women with a history of nephrolithiasis who underwent an oral radioactive calcium assay found that increased calcium intake reduced the likelihood of nephrolithiasis by 45–54% ($p = 0.03$) [288].

In 2010, a meta-analysis showed a possible increase in atherosclerosis in women receiving calcium supplements [289]. However, a recent meta-analysis including 18 RCTs (n = 63,564) reported no effect of calcium supplementation with or without vitamin D on coronary heart disease or all-cause mortality risk in elderly women [290]. This meta-analyses described cardiovascular events clinically verified by hospital records or death certificates. The RR was 0.96 (95% CI: 0.91–1.02; $p = 0.180$) for all-cause mortality, 1.02 (95% CI: 0.96–1.09; $p = 0.510$) for coronary heart disease events; 1.08 (95% CI: 0.92–1.26; $p = 0.320$) for myocardial infarction; 1.09 (95% CI, 0.95-1.24; $p = 0.22$) for angina pectoris and acute coronary syndrome and 0.92 (95% CI, 0.73-1.15; $p = 0.46$) for chronic CHD [290]. A 32% self-reported false positive rate for myocardial infractions was reported in the Women's Health Initiative (WHI) study and in the Nurses' Health Study [291, 292]. Lewis et al. [293] reviewed the data from RCTs on calcium supplementation and errors in self reporting of myocardial infarction. This review included studies used in the Bolland et al. [289] meta-analyses and a few others and

concluded that calcium tablets increase the incidence of gastrointestinal events, which may be mistaken by participants as myocardial infarction leading to reporting bias in studies of calcium supplementation [293]. On the contrary, a recent mendelian randomization analyses found a casual evidence for effects of high serum calcium with conditions related to renal function and CVD risk [283].

A suggested mechanism by which supplemental calcium may elevate CVD risk is through the acceleration of vascular calcification induced by acute elevations in circulating serum calcium levels [294]. In a study conducted at Purdue university [295] using a relevant animal model (Ossabaw miniature swine) which has translation relevance to humans [296] provided mechanistic insights on the relationship between calcium intake and heart health. The animals were fed an atherogenic diet with adequate calcium or high calcium for 6 months. Following five months of feeding, all animals were dosed intravenously with 41Ca, a rare isotope that can be measured in serum and tissues with sensitivity of 10^{-18} mol/L by accelerator mass spectrometry in order to determine early coronary artery calcium deposition. High calcium diets approaching the equivalent of the upper tolerable levels for humans (approx.2000-2500mg/day) did not result in any worsened indices of cardiovascular function, coronary artery plaque burden, or coronary artery calcification compared to adequate calcium intakes [295]. The results were similar whether the predominant source of calcium in the feed was dietary calcium or calcium carbonate supplement.

The weight of these collective findings supports the notion that high consumption of calcium does not increase the risk of cardiovascular events or the risk of kidney stones.

1.7 Vitamin D and Bone Health

1.7.1 Vitamin D- Requirements, Function and Metabolism

Vitamin D is a prohormone that regulates calcium and phosphate metabolism. It is synthesized in the human body through the action of sunlight where UVB irradiation converts 7-dehydrocholesterol to provitamin D_3 that at body temperature isomerizes to

vitamin D_3. Vitamin D can also be sourced from the diet from either animal source which is vitamin D_3 (cholecalciferol) or plant sources vitamin D_2 (ergocalciferol) [297]. IOM recommends a vitamin D intake of 600 IU per day for the general, healthy population aged 1–70 years, while for individuals older than 70 years this was increased to 800 IU per day [298]. A supplement of 800 IU per day is recommended to achieve serum 25(OH)D levels of 50 nmol/L or greater.

In the circulation, vitamin D and its metabolites are largely bound to the vitamin D binding protein (DBP). Vitamin D is activated by two step hydroxylation reactions catalyzed by specific cytochrome P450 (CYP) enzymes. The first hydroxylation at the carbon 25 position takes place in the liver and is mediated by the enzyme vitamin D-25 hydroxylase (CYP2R1) to form the prohormone 25-hydroxyvitamin D, 25(OH)D. The liver is the major site of synthesis for plasma 25(OH)D but the enzyme CYP2R1 is also expressed in a variety of other tissues and hence the synthesis of 25(OH)D can occur locally throughout the body [299]. Of all the vitamin D metabolites, 25(OH)D is the most abundant form, has the longest half-life, approximately 2 to 3 weeks, reflects both skin synthesis and dietary intake and is not under homeostatic control. It is thus regarded as the biomarker of vitamin status [37]. The second hydroxylation at the carbon one position occurs in the kidney where the biologically active metabolite $1,25(OH)_2D$ is produced in a reaction catalyzed by the enzyme CYP27B1.

A third step in vitamin D metabolism is hydroxylation at the carbon 24 position by the 25-hydroxyvitamin D-24-hydroxylase (CYP24A1) enzyme. This enzyme is mainly responsible for catabolism of vitamin D metabolites through its multicatalytic activity to form calcitroic acid which is excreted via the kidneys. The combined activities of the renal CYP24A1 and renal CYP27B1 enzymes are responsible for determining plasma levels of $1,25(OH)_2D$ [300]. $1,25(OH)_2D$ acts by binding to VDR and plays a principal role in the maintenance of plasma calcium and phosphate homeostasis and influences bone mineral content according to the needs of plasma calcium. This is the well-defined classical endocrine pathway of vitamin D [300]**.** The endocrine activities of $1,25(OH)_2D$ to maintain plasma calcium and phosphate levels include stimulation of intestinal calcium and phosphate absorption, reduction of calcium excretion through actions on the kidney and increasing bone resorption to release calcium and phosphate from the

bone [301].

1.7.2 Skeletal Effects of Vitamin D Deficiency

Vitamin D status is assessed by measuring the serum levels of prohormone 25(OH)D reflects both cutaneous synthesis and dietary intake [302]. In general, vitamin D levels less than 25 nmol/L are defined as deficient, levels between 25-50 nmol/L are defined as insufficient and levels greater than 50 nmol/L are defined as sufficient or optimal levels [302].

Deficiency of vitamin D manifests clinically as two separate disorders depending on serum 25(OH)D levels; osteomalacia (mineralization defect) and osteoporosis (bone fragility). Clinical evidence indicates that vitamin D deficiency at a level less severe than that which causes osteomalacia contributes to osteoporosis [132]. These clinical conditions are discussed below.

1.7.2.1 Vitamin D Deficiency: A Cause of Osteomalacia

Osteomalacia in adults and rickets in children, is regarded as the index disease of vitamin D deficiency which results primarily from the malabsorption of calcium and phosphorus. Inadequate vitamin D intake or sunlight exposure results in reduced serum 25(OH)D levels, thereby limiting the production of adequate serum $1,25(OH)_2D$ levels. In such conditions, renal CYP27B1 enzyme is markedly upregulated (around 40 fold) to maintain plasma $1,25(OH)_2D$ levels which ensures maintenance of intestinal calcium absorption, renal tubular reabsorption of calcium and stimulation of calcium and phosphate release from the bone into the plasma pool even at lower levels of 25(OH)D [303, 304]. Clinical evidence suggests that serum $1,25(OH)_2D$ levels and intestinal calcium absorption are not affected until 25(OH)D levels fall below 20 nmol/L [303, 305]. However, when 25(OH)D levels fall below 20 nmol/L, substrate levels for the kidney enzyme CYP27B1 are limiting and plasma $1,25(OH)_2D$ levels fall with simultaneous reduction in intestinal calcium absorption and significant increase in serum PTH levels [303]. Additionally, increased PTH levels lower the renal threshold for phosphorus, which, together with reduced phosphorus absorption, produces hypophosphatemia. Low serum phosphorus and calcium levels causes mineralization defect of the newly laid

bone (osteoid). The under mineralized osteoid does not provide structural support for the skeleton and increases the risk of fracture [306]. Thus, serum 25(OH)D levels less than 20 nmol/L results in osteomalacia or rickets and predisposes individuals to a higher risk of fractures. Previously, hip fractures in elderly patients with low vitamin D status was thought to be the result of osteomalacia rather than osteoporosis [132, 307, 308]. However, several studies have reported that mean serum 25(OH)D levels in hip fracture patients is significantly above 20 nmol/L and is reported to be between 30-50 nmol/L indicating that osteoporosis has a completely different etiology [132, 222, 225, 309].

1.7.2.2 Vitamin D Inadequacy: A Cause of Osteoporosis

The skeletal effects of moderate vitamin D deficiency in adults or elderly subjects are mainly caused by elevated PTH levels leading to high bone turnover [22]. In the Multiple Outcomes of Raloxifene Evaluation study, the patients with vitamin D deficiency (25(OH)D < 25 nmol/L and between 25 to 50 nmol/L) showed significantly higher serum PTH levels as compared to patients with 25(OH)D levels above 50 nmol/L. Treatment with vitamin D resulted in significant decline in serum PTH levels ($p < 0.001$) [310]. Consistent with this, significant reductions in serum PTH, CTX-1 and osteocalcin levels ($p < 0.001$) and increases in BMD at all sites (lumbar spine, total hip, femoral neck, and femoral trochanter, $p < 0.001$) with increasing 25(OH)D levels were reported in a large prospective study involving 7441 postmenopausal women from 29 countries [311]. A threshold for the positive relationship between 25(OH)D and BMD at different sites was observed at a 25(OH)D level of 50 nmol/L while PTH and 25(OH)D levels were negatively associated and no plateau for this relationship was observed up to 25(OH)D levels of 100 nmol/L [311].

Consistent with this, the longitudinal aging study Amsterdam reported significantly lower PTH levels with higher 25(OH)D levels up to values of 100 nmol/L and higher vitamin D levels, with no plateau observed. BMD at the total hip and femoral trochanter was significantly higher in people with higher 25(OH)D levels with a threshold observed at 50 nmol/L [312]. Bischoff-Ferrari et al., proposed a threshold level for 25(OH)D at 75 nmol/L for BMD, lower-extremity function and risk of falls and fractures [30]. In the National Health and Nutrition Examination Survey (NHANES), a significant

positive association between serum 25(OH)D levels and total hip BMD in young and older adults was observed [313]. In a cross-sectional study of 2310 adults, PTH levels were lowest in the people with 25(OH)D greater than 45 nmol/L. Interestingly, this study also showed that a sufficient 25(OH)D levels can maintain lower PTH concentrations with calcium intake less than or equal to 800 mg/d but a higher calcium intake of more than 1200 mg/d was not sufficient to maintain lower PTH levels if vitamin D status is insufficient highlighting the need to maintain adequate serum 25(OH)D levels [314].

Despite all the positive association data between 25(OH)D levels and BMD and fracture risk, RCTs of vitamin D supplementation on BMD [36, 240, 315] and fracture risk [27, 316] have failed to demonstrate an effect of vitamin D supplementation alone. Most of the meta-analyses have reported null findings [34, 232, 239] while two meta-analyses have shown positive effect of vitamin D in reducing fracture risk [233, 234]. Bischoff-Ferrari et al., reported a 20% (RR: 0.80, 95% CI 0.72-0.89; n = 33,265) reduction in nonvertebral fractures and 18% (RR 0.82, 95% CI 0.69-0.97, n = 31,872) reduction in hip fracture when vitamin D doses were between 482-770 IU/d in older individuals (age > 65 years). Fracture reduction was not observed when supplemental vitamin D intake was 400IU/day or less. Further, nonvertebral fracture reduction was independent of age or whether the participants were community dwelling or institutionalized. At higher vitamin D doses, the effect of vitamin D on anti-fracture efficacy was independent of calcium intakes [234]. Later, in 2012 these authors performed another pooled analyses considering actual intake of participants rather than relying on randomly assigned dosage. The findings suggested that vitamin D doses greater than 800 IU/day were most beneficial in reducing hip and non-vertebral fractures independent of calcium intake. Further, the authors proposed that 25(OH)D levels above 60 nmol/L are required for fracture prevention. However, reduction in any fracture was observed only in the intention to treat analyses [233]. It is important to note that several studies have shown that bolus doses of vitamin D, both annually (such as 500,000 IU) or monthly (60,000IU), are associated with higher propensity for falls and fractures [317, 318].

To understand the effectiveness of vitamin D to maintain or improve bone health, it may seem important to study actions of vitamin D independent of calcium intake, but mechanistically this may be less relevant as these nutrients act in concert to

maintain skeletal health [319]. Furthermore, as stated by Heaney *"an excessively reductionist approach may be intrinsically inappropriate in any nutritional study, because most nutrients act in concert with one another, and the attempt to establish efficacy for one apart from the other, as if they were drugs, may be misguided"* [319]. Clinical and epidemiological studies that fail to take this approach of combining calcium and vitamin D often produce null findings and meta-analysis or systematic reviews that fail to exclude such studies also result in null findings. In the recent meta-analyses by Bolland et al., [33] all studies using combined vitamin D and calcium supplementation were excluded and authors meta-analyzed the results of vitamin-D-only supplementation. The authors concluded what has been already established by several past meta-analyses [231, 235] i.e. vitamin D alone has no effect on fracture risk reduction. In 2014, the authors themselves concluded that vitamin D co-administered with calcium reduced the risk of hip fracture in institutionalized individuals [34] which corroborates with the other meta-analyses [20]. In the recent vitamin D assessment study (ViDA), which included 5110 participants, aged 50-84 years, mainly from primary care practices in Auckland, New Zealand, involved supplemenatation with large doses of vitamin D3 equivalent to about 3000 IU per day (100,000 IU vitamin D3 monthly) for 3.4 years. The study showed no reduction in the risk of non-vertebral fractures and falls [242]. As explained above, studies in the past have already shown that bolus doses of vitamin D is associated with increased risk of falls [318, 320].

Hence, in the following sections (section 1.8), we present clinical evidence of combined calcium and vitamin D supplementation on BMD and anti-fracture efficacy. Also, we will discuss briefly the null effects observed in certain trials.

1.7.3 Vitamin D Toxicity

Vitamin D is a fat-soluble nutrient; hence excess levels are not excreted but stored in the body and cause vitamin D toxicity. Doses of vitamin D between 20,000 and 40,000 IU/d taken on a long-term basis results in serum 25(OH)D levels exceeding 500 nmol/L. At these supraphysiological levels, serum 25(OH)D can bind directly to VDR simulating the effects of $1,25(OH)_2D$ which may cause hypercalcemia [321, 322]. IOM defines 4000 IU/day as the upper tolerable limit for vitamin D for adults [298]. Serum

25(OH)D levels greater than 125 nmol/L have been found to be associated with increased mortality rates [323, 324].

1.8 Calcium-Vitamin D and Bone Health

1.8.1 Calcium-vitamin D- Effect on BTMs and BMD

A meta-analysis of 23 trials (n = 41,419) reporting BMD as an outcome reported a 0.54% ($p < 0.001$) reduction in bone loss at the hip and 1.19% ($p < 0.0001$) reduction in bone loss at the spine with calcium supplementation and calcium in combination with vitamin D [236]. Zhu et al., reported maintenance of bone mass at the hip in older Australian women, after one-year supplementation with calcium (1200 mg/d) or calcium plus vitamin D (1200 mg/d calcium and 1000 IU/d vitamin D) as compared to placebo. However, the effect of supplementation was only maintained in the group that received both calcium and vitamin D at three ($p = 0.01$) and five years ($p = 0.05$) suggesting that vitamin D has additional benefit in preventing bone loss [325]. This could possibly be due to the combined effectiveness of calcium and vitamin D supplementation to suppress bone turnover as only in the calcium-vitamin D group, serum PTH, bone ALP and deoxypyridinoline (DPD) levels were suppressed significantly [325]. Dawson-Hughes et al., reported a significant reduction in bone loss at femoral neck ($p = 0.02$), spine ($p = 0.04$) and total body BMD ($p < 0.001$) in 389 community dwelling men and women consuming 500mg calcium and 700 IU vitamin D as compared to placebo group after one year of supplementation. This was a three-year supplementation trial and the effect was significant only for total body BMD in the second and third year. The initial effects of supplementation at the hip and spine observed at one year were maintained but not increased during the subsequent two years of the study [326]. Further, three years supplementation with calcium-vitamin D resulted in a significant reduction in osteocalcin levels both in men (9%, $p < 0.005$) and women (14%, $p < 0.005$) from baseline indicating reduced bone remodelling rate [326].

1.8.2 Calcium-Vitamin D and Fracture Risk

The majority of RCTs and meta-analyses that examined the effects of calcium

and vitamin D supplementation together have shown a reduction in fracture risk in elderly people provided that adequate dietary calcium and vitamin D doses were given, sufficient patient compliance (at least 80%) was ensured and the participants were deficient at baseline [20, 24, 25, 231, 233, 235].

Dawson Hughes et al., reported a 50% reduction (RR 0.5; 95% CI, 0.2-0.9; p = 0.02) in the risk of vertebral fracture in community-dwelling men and women supplemented with 500 mg calcium and 700 IU of vitamin D daily for three years [326]. Chapuy et al. reported 30% lower hip and nonvertebral fractures in elderly institutionalized women who were treated with 1200 mg calcium and 800 IU of vitamin D per day three years [24, 25]. Weaver et al., in a meta-analysis of eight studies including 30,970 participants (192 hip fractures and 2231 total fractures cases) showed vitamin D and calcium supplement together lead to a 15% reduction in risk of all fractures and 30% reduction in hip fracture risk, both reaching statistical significance [20]. Yao et al., recently, conducted a meta-analysis to assess the risk of fracture associated with differences in 25(OH)D levels in observational studies and the risk of fracture associated with supplementation with vitamin D alone or combined with calcium in RCTs. In the meta-analysis including 6 RCTs (49282 participants, 5449 fractures, 730 hip fractures), authors found a 6% reduction in risk of any fracture (RR 0.94; 95% CI 0.89-0.99) and 16% reduction in risk of hip fracture (RR 0.84, 95% CI 0.72-0.97) with combined calcium and vitamin D supplementation as compared to control (placebo or no treatment). The vitamin D dosage in the trials included was either 400 or 800 IU/day and the calcium dosage was between 800-1200 mg/day. In the meta-analyses including 11 observational studies (39141 participants, 6278 fractures, 2367 hip fractures), each 25 nmol/L increase in 25(OH)D levels was associated with a 7% reduction in the risk of any fracture (RR: 0.93; 95% CI 0.89-0.96) and 20% reduction in the risk of hip fracture (RR: 0.80; 95% CI 0.75-0.86) [327].

The trials that showed no effect of calcium and vitamin D on fracture risk reduction mostly had methodological differences, such as lack of patient compliance, under-powering of the study to detect fracture outcomes, whether supplementation included both calcium and vitamin D or just one of these nutrients, dosage, source (e.g. vitamin D_2 vs, D_3; vitamin D_2 is generally considered to have lower molar potency than

$D_{3)}$ [234], the design of the study (with placebo or no placebo), baseline calcium or vitamin D intake, administration of hormone replacement therapy prior to or during the study and heterogeneous populations. Hence, variable outcomes from meta-analyses or systematic reviews have arisen due to analysts combining data from such trials with methodological issues in their analyses. This is a complex scientific issue which has been debated and reviewed extensively [20, 35].

One of the best examples of the complexities in analyzing such data is the experience of a major trial, the WHI. It was the largest and longest double blind, RCT on calcium and vitamin D supplementation and risk of fractures [282]. The study included 36,282 postmenopausal women randomized to either 1000 mg calcium carbonate with 400 IU of vitamin D3 daily or placebo group for seven years. The trial included women taking vitamin D and calcium supplements before recruitment both in the placebo and experimental groups as well as women not taking these supplements in both groups. After a seven-year intervention period, treatment resulted in a small but significant improvement in hip bone density but no significant reduction in risk of hip fracture [282]. Later in 2013, Prentice et al.[237] conducted a post hoc analysis of the WHI clinical trial data focusing on participants who were not taking any supplements at baseline (n = 7584) and who adhered to the treatment (n = 7718). The authors reported a 38% reduction in fracture risk (HR 0.62; 95% CI 0.38-1.00) in those subjects who were not taking supplements at recruitment and then assigned to supplements for the trial versus those not taking supplements at recruitment and assigned to the placebo group [20, 237]. In the Tang et al., meta-analyses involving 52,625 participants from 17 trials (discussed above in section 1.6.5), the fracture risk reduction was significantly greater (24%, $p < 0.001$) in trials in which compliance rate was high (80% or more) and the treatment effect was better with calcium doses of 1200mg or more than with doses less than 1200 mg (0.80 versus 0.94; $p = 0.006$) and with vitamin D doses of 800 IU or more than with doses less than 800 IU (0.84 versus 0.87, $p = 0.03$) [236]. A further meta-analysis including data from 68,517 patients showed that vitamin D alone had no beneficial effect on risk of fracture. Trials of vitamin D combined with calcium showed 8% ($p = 0.025$) reduction in the risk of all fractures and a borderline reduction (16%, $p = 0.07$) in the risk of hip fractures. However, when studies using 400 IU vitamin D dosage

with calcium were included, highly significant 26% (p = 0.005) reduction in hip fracture incidence was observed [231]. This does not mean that 800 IU dosage was inferior but was due to fact that the trials with higher dosage attempted to provide fracture prevention at a threefold higher risk level for hip fractures than did studies using the lower dose (400 IU dose: treated 2.4/1000 person year; untreated 3/1000 person year; HR = 0.74, p = 0.005 vs 800 IU dose; treated 10.3/1000 person year; untreated 9.2/1000 person year; HR = 1.3, p = 0.19) [231].

Thus, updated WHI [237], Tang et al [236] and the DIPART study [231] all the three highlight the importance of dosage and compliance to demonstrate the anti-fracture efficacy of calcium and vitamin D. With regards to vitamin D dosage, a consistent and significant benefit has been established at doses greater than 800 IU per day [233, 234] and with 1000 mg calcium [20, 233].

United States Preventive Task Force (USPTF) issued recommendation statement in 2013 and further in 2018 where in they have recommended against daily supplementation with 400 IU or less of vitamin D3 and 1000 mg or less of calcium for the primary prevention of fractures in community-dwelling, postmenopausal women. This report is based on the results obtained from eight RCTs on vitamin D or calcium, or combined supplementation for the primary prevention of fractures. Four trials evaluated vitamin D supplementation alone (n = 10,606), two trials evaluated calcium supplementation (n = 339), and two trials evaluated combined vitamin D and calcium supplementation (n = 36,727) [26]. Of the four vitamin D trials, two trials evaluated a dosage of less than 400 IU and did not find any difference in fracture outcomes. The two other trials consisted of administering large bolus doses of vitamin D and one of them was not even designed to assess fracture outcome but cardiovascular events. It has already been explained above that vitamin D supplementation alone does not reduce fracture risk and that bolus doses of vitamin D may actually increase the propensity of fall [231, 234, 235, 318, 320, 328]. Two calcium supplementation studies included calcium supplementation at 1200 mg and 1600 mg dosage levels. These two studies did show a modest reduction in fractures risk, however, the USPTF researchers reported that none of them were adequately powered to detect the differences. There are several well-designed calcium supplementation studies which have shown statistically

signifcant reduction in bone loss [3, 15, 258, 272-274] and the risk of fractures [277-280]. These studies have already been discussed in detail in section 1.6.5 and 1.6.6.

Only two studies with combined calcium and vitamin D supplementation were included in the USPTF report. One of them was the original WHI trial published in 2006 [282]. The issue with the original analyses has been described above in this section and the researchers of the WHI trial have themselves published an updated report in 2013 [237]. The second trial included in the USPTF report is the Dawson-Hughes et al study [326] (discussed above in section 1.8.1 and 1.8.2). This study reported that addition of 500 mg of calcium per day, along with 700 IU of vitamin D3, reduced the incidence of nonvertebral fractures in both men and women while no effect on hip fractures was found. Of the 37 subjects who had nonvertebral fractures, 26 were in the placebo group and 11 were in the calcium–vitamin D group ($p = 0.02$). The USPTF researchers negated the significant effect observed in this study on nonvertebral fracture on the basis that nonvertebral fracture assessment was not the primary aim of the study. While this is a reasonable argument, the primary aim of the Dawson-Hughes trial was to assess changes in BMD over a period of 3 years and not was not designed to assess the risk of hip fractures. This study consisted of 389 participants with one case of hip fracture reported in the placebo group and none in the treatment group over the three-year study period [326]. Notable, the USPTF recommendations apply only to community-dwelling and asymptomatic adults not known to have osteoporosis or vitamin D deficiency or to be at high risk of fracture.

Two recent meta-analyses have shown no impact of calcium and vitamin D supplementation as compared to placebo in prevention of fracture in community dweelling individuals [28, 29]. In the Zhao et al., meta-analyses all RCTs studies with calcium alone, vitamin D alone or their combination, with or without a placebo group were included. Further, vitamin D trials with bolus doses were also included and authors did not assess participant complaince to the supplements. The control group patients in some of the studies may be taking supplements outside the study. Further, the duration of included trials varied from 4 months to 7 years which could have influenced the results [28]. In the other systematic review and network meta-analyses, Hu et al., evaluated the optimal concentration of vitamin D, calcium or their combination required

to prevent fractures in community-dwelling older people. The authors reported no affect of calcium alone or calcium in combination with vitamin D to prevent fractures. The inclusion of all the trials with calcium alone or vitamin D alone or combined calicum-vitamin D and trials with bolus doses of vitamin D might have influenced the results as discussed above. Further, this meta-analyses did not address the issue of patient complaince [29]. The original WHI data [282] was included instead of the updated analyses [237] and in the RECORD trial included in this analyses [329] only 42% of the participants were taking calcium or vitamin D supplements at two years. As both these trials were substantially larger than all the other studies included in this meta-analyses, patient non-compliance in these trials may have influenced the results.

Thus, the field of calcium and vitamin D supplementation has clearly ongoing controversies that still needs to be resolved in order to more effectively guide clinical practice on the use of calcium-vitamin D supplemention as a public health strategy.

We believe in order to advance the field of calcium and vitamin D further; it is important to gain mechanistic insights on how vitamin D impacts bone health and reduces fracture risk in combination with calcium. Elucidating the mechanism of action of vitamin D could help in identifying relevant structural and metabolic endpoints that could be confirmed in clinical trials at some stage. Studies undertaken in this thesis provides data to bridge some of the existing knowledge gaps.

1.9 Aim 1 and Hypothesis

A clinical paradox to the endocrine paradigm of vitamin D activities is that all the beneficial effects of vitamin D on BMD and fracture risk (when co-administered with calcium) are associated with serum 25(OH)D levels and not the biologically active form serum $1,25(OH)_2D$ levels [232, 330]. These data raise the notion that serum 25(OH)D levels have a role in improving bone health beyond providing substrate for plasma $1,25(OH)_2D$ levels to maintain intestinal absorption of calcium. Seminal discoveries that the three major bone cell types, osteoblast, osteoclasts and osteocytes, express genes for vitamin D metabolizing enzymes i.e. CYP27B1, CYP24A1 [132, 331, 332] and VDR suggests [333] that bone cells have the complete machinery to synthesize $1,25(OH)_2D$

locally within the bone. These observations suggest that vitamin D may exert autocrine or paracrine activities within bone tissue which are independent of its endocrine actions i.e. circulating $1,25(OH)_2D$ levels. Studies carried out by our group have provided intriguing insights on the autocrine-paracrine activities of vitamin D [301, 332, 334-344]. Preliminary data obtained from transgenic mouse models and dietary animal studies suggest that in rodents when calcium levels are adequate, $1,25(OH)_2D$ synthesized within bone increases bone mineral status through autocrine-paracrine activity [301, 345-347]. Based on these observations, one of the studies described in this thesis was based on the hypothesis that adequate serum 25(OH)D levels (>50 nmol/L) may be metabolized locally within the bone thereby leading to improved bone microarchitectures.

1.9.1 Aim 1

To investigate the associations between vitamin D metabolites ($1,25(OH)_2D$) and 25(OH)D), PTH, BTMs and femoral bone structure of elderly hip fracture patients.

1.10 Obesity and Bone Health

Clinical and mechanistic studies have demonstrated important interaction between the skeleton and adipose or fat tissue. Both these tissues have a common progenitor (MSC) which can differentiate into an osteoblast or an adipocyte. The peroxisome proliferator–activated receptor-δ (PPARδ) plays an important role in initiating adipogenesis and inhibiting osteoblastogenesis [114]. Several drugs such as glucocorticoids act as ligands for PPARδ and influence the fate of MSC and promote adipogenesis at the expense of osteoblastogenesis [348]. Additionally, disorders associated with obesity have been linked to prevalent chronic diseases such as atherosclerosis, diabetes mellitus and osteoporosis [349]. Further, adipose tissue secretes peptides, known as adipokines, that act in autocrine, paracrine and endocrine pathways to influence whole body energy homeostasis [350]. Thus, both structurally and functionally, there exists a close relationship between adipocytes and osteoblasts.

Obesity has traditionally been considered to have a positive effect on bone as

overweight/obese individuals generally present with higher BMD; yet the risk of fractures is not low in this population [112, 351]. More recent findings suggest that bone quality is compromised in obese individuals [348, 352-357]. Thus, the potential interplay between adiposity and bone health is complex and needs to be investigated from several aspects such as effect of BMI and fat mass content and fat distribution on BMD and fracture risk.

Overweight and obesity refers to abnormal or excessive fat accumulation that presents a risk to health. It is defined by body mass index (BMI) which is calculated as weight divided by the square of height and is expressed as kg/m^2. BMI is the basis of the current categorization system for obesity and is widely used across disciplines ranging from epidemiological studies to individual patient assessment. The various BMI categories and respective BMI cut-offs are: underweight less than 18.5 kg/m^2, normal weight 18.5-24.9 kg/m^2, overweight between 25-29.9 kg/m^2, obese > 30 kg/m^2 [358].

Studies using sophisticated techniques such as MRI, quantitative computed tomography (QCT) and Lunar iDXA which can differentiate between various fat fractions suggests that increases in abdominal visceral fat confer an independent risk to bone health [354, 355, 357]. The effect of visceral fat on fracture risk is discussed in section 1.10.4

1.10.1 Higher BMI Is Associated with Higher BMD

Initially, higher BMD observed in obese individuals was thought to be an artifact due to overlying soft tissue. Further, the coefficient of variation reported between repeat BMD measurements is higher in obesity [359]. However, studies using pQCT which are not affected by overlying soft tissue and provide separate information about cortical and trabecular compartments confirm that obese individuals have higher volumetric BMD, higher cortical BMD, higher trabecular BMD and greater trabecular number at the distal radius and distal tibia [351, 360]. There are multiple reasons for higher BMD in obese individuals such as greater mechanical loading which stimulates bone formation by decreasing apoptosis and increasing proliferation and differentiation of both osteoblasts and osteocytes [361] and activation of the intracellular signaling

Wnt/b-catenin which is a key pathway implicated in bone formation [362]. Obesity is associated with higher levels of serum estrogen and pancreatic hormones such as insulin, amylin, and preptin that are anabolic to bone [115].

1.10.2 Higher BMD may not Compensate for Increased BMI

It has been postulated that although obesity may increase BMD, the increase in bone strength may not be in proportion to the increase in BMI and fall impact forces [363]. In a matched case-control study in a French cohort (OLEFY) of obese postmenopausal women, it was shown that higher values for BMD, trabecular microarchitecture and bone strength variables, were not in proportion to their higher BMI compared to normal weight women leading to relative bone fragility [360]. Thus, in obese individuals, higher BMD is not commensurate with their increased body weight and the load-to-strength ratio rises significantly, leading to low-trauma fractures. Similar findings have been reported in The Study of Women's Health Across the Nation (SWAN) [363] and in a subset of the WHI-observational cohort [364]. A recent meta-analysis examining the influence of adipose tissue on bone mass demonstrated divergent relationships between BMD and total fat mass or adiposity. Absolute fat mass correlated positively with BMD while relative adiposity (% fat mass to total weight) correlated negatively with BMD and these relationships were sex and age dependent [365]. All these data confirms that the increase in BMD with higher BMI may not be sufficient relative to body weight and fall forces especially during the years when bone metabolism is in a state of rapid turnover as in older years [365].

Some studies reveal that both lean mass and fat mass are equally associated with BMD [366, 367] whereas others showed that lean mass is a stronger predictor of BMD than fat mass [360, 368-370] and yet few others have demonstrated that total fat mass is a stronger predictor of BMD [371-373]. Taken together, these studies suggest that the relative contribution of lean and fat mass on BMD could be dependent on sex, ethnicity, and age. In this regard, findings from a meta-analysis are useful as they suggest that correlation between lean mass and BMD in the femoral neck was greater than that of fat mass in men and women combined. Lean mass accounted for 21% of the variability while fat mass accounted for 8% of the variability in total body BMD [374]. However, the

associations between lean mass and BMD appears to be sex, age group and skeletal site specific [374].

With advancing age there is a natural decline in muscle mass relative to body mass [375]. A combination of high fat mass along with low muscle mass in older obese people is clinically defined as obese sarcopenia to reflect the direction of pathological pathway i.e. obesity possibly leading to muscle damage [376]. Aging and obesity are independently associated with inter and intramuscular fat infiltration resulting in reduced muscle strength and mobility [377, 378] through different mechanisms. Loss of muscle function or mass with age is referred to as sarcopenia which also results in increased propensity to falls and fractures. However, the condition is aggravated or more complex in obese individuals where inflammation associated with obesity is the cause of muscle damage and higher risk of falls and fractures [376]. The underlying postulated mechanism for obese sarcopenia is sustained inflammation of adipose and skeletal muscle tissue which results in significant muscle damage. Obese adipose tissue is characterized by increased secretion of molecules such as adipokines, cytokines, myokines that create a chronic low grade systemic inflammation, disturbed capacity to store lipids which results in ectopic fat accumulation, insulin resistance and muscle dysfunction [376]. A longitudinal study in community dwelling older Australian adults showed that sarcopenic obese people are at a greater risk of osteoporosis and non-vertebral fracture as compared to obese alone individuals [379].

1.10.3 Higher BMI is Associated with Site-Specific Higher Fracture Risk

One of the earliest evidence that obesity is not protective against fractures was highlighted in a study from a Fracture Liaison clinic in UK where in a high prevalence of overweight and obesity (28%) was recorded in post-menopausal women presenting with low-trauma fractures and majority of them (more than 60%) had normal BMD [380]. This was further investigated in the Global Longitudinal study of Osteoporosis in Women (GLOW), a large, prospective, multinational cohort study in 60,393 postmenopausal women aged 55 years or greater. During the 2 years of study follow-up, fractures rates were similar between obese and non-obese women [381]. Consistent with these findings, in the Osteoporotic Fractures in Men study (MrOS), 68% of incident fractures

and 62% of hip fractures occurred in men who were overweight or obese [382]. In the NHANES survey, 59% and 46% of hip fractures occurred in men and women, respectively who were overweight or obese [383]. These and many other similar studies have challenged the long-held opinion that obesity is protective for fractures.

On the other hand, low BMI is an established risk factor for hip fractures. In 2005, De Laet et al., in a meta-analysis of twelve prospective cohort studies comprising of 60,000 men and women, reported that low BMI was a significant risk factor for hip fracture. After adjustment for BMD, a 5-unit reduction in BMI from 25 kg/m^2 to 20kg/m^2 resulted in a 42% higher risk of hip fracture (RR = 1.42, 95% CI 1.23-1.65). However, a 5-unit increase of BMI from 25 kg/m^2 to 30 kg/m^2 was not associated with any change in hip fracture risk (HR = 1.00, 95% CI 0.82-1.21) indicating that leanness is a risk factor for hip fracture but overweight may not be protective [140]. This was later confirmed in 2014 in a larger meta-analysis consisting of prospective cohort studies including 398,610 women (age range 20-105 years, mean age 63 years) which showed that women with BMI of 15 kg/ m^2 had a 60% higher risk of hip fracture compared to those with BMI of 25 kg/m^2 (HR =1.41, 95% CI 1.16-1.72), while there was no difference in hip fracture risk between women with BMI of 35 kg/m^2 and BMI 25 kg/m^2 (HR = 0.99, 95% CI 0.86-1.15), after adjustment for BMD [139]. Hence, low BMI is included in fracture risk prediction algorithms such as FRAX and Garvan calculators and people with BMI less than 20 kg/m^2 are at highest risk [139].

The influence of BMI on fracture risk has been reported to be site-specific. The site-specific fracture occurrence in obese individuals may be related partly due to a greater fat padding surrounding some areas such as hip that reduces the impact of falling while other sites are more exposed due to less fat such as ankle or humerus [348]. Most of the available evidence supports a lower risk of wrist fracture in obese adults and higher risk for proximal humerus (rate ratio 1.28, p = 0.018 , upper leg (excluding hip) (OR 1.7, 95% CI 1.1-2.5) and ankle fracture (OR 1.5, 95% CI, 1.2-1.9) [112, 139, 384].

Data on the relationship between BMI and risk of vertebral fractures are somewhat conflicting. Some studies have found higher risk in obese women [188, 385-387] whereas others have not [112, 384]. Bachmann and colleagues in a cross-sectional

study involving 176 premenopausal women across a broad spectrum of BMI (14-45.3 kg/m^2) provide some insights on the variable effect observed on risk of vertebral fractures. The study showed that women with BMI of 17.1± 1.1 kg/m^2 (anorexia nervosa) have lower vertebral strength and hence are at a higher risk of fracture (estimated fracture risk) during activities like holding and bending. On the other hand, overweight and obese women (BMI>28 kg/m^2) had higher vertebral fracture risk (estimated fracture risk) for standing, holding, and lifting because of higher applied loads from higher body weight, in spite of having similar vertebral strength as normal weight women [356]. Similar findings have been reported in the recent cross-sectional analysis of 342 participants from the NTFS birth cohort. This study showed that obese women were more likely to have at least one vertebral fracture (OR = 2.65, p = 0.025) and at least one grade 1 vertebral deformity (OR = 4.39, p = 0.005) as compared to normal weight women. obesity is a risk factor for prevalent vertebral fractures, although absolute BMD was higher in obese individuals [386].

With regards to site-specific effects of BMI on fracture risk, no site-specific BMD deficits are reported to account for the site-specific differences observed. The site-specific differences and increased risk of fractures in overweight and obese people could be partially explained by pattern of fall [388] and the type of dominant bone present: cortical or trabecular bone [389]. Fall patterns may be different in obese individuals compared to non-obese individuals. Obese individuals tend to fall backward or sideways rather than forward which may protect the wrist from the impact [388]. On the other hand, the additional body weight in overweight/obese people may increase the fall force much more than what can be accommodated by the arm and increase the risk for humerus fractures [388, 390]. The exact reason for increased fracture risk at the ankle and leg in individuals with higher weight are unknown but excessive stresses associated with bending or torsion may be involved [388].

With regards to the type of dominant bone present, data suggest that obese individuals have lower cortical vBMD and higher values for trabecular bone parameters [389, 391]. Sites like the humerus have higher cortical bone and hence may be more prone to fractures while wrist and hip regions have significant trabecular bone and hence may be less susceptible to fractures in obese women. However, the data is not

that simple or straight forward. Recent studies using central adiposity such as waist circumference (WC) and waist-hip ratio as a marker of obesity suggest an increased risk of hip fracture [392] and is discussed further in the next section.

1.10.4 Central Adiposity and Fracture Risk

1.10.4.1 Central Adiposity Measured as Waist Circumference and Fracture risk

Assessment of obesity using BMI gives an indication overall fat mass but does not discriminate between mass contributed by lean mass from fat or adipose tissue mass. Further, over and above adipose tissue mass per se, BMI classification provides no information on the pattern of fat or adipose distribution or the kind of adipose tissue which is more closely linked with metabolic health. Central adiposity is defined as the amount of fat around the abdominal area, also known android obesity. Epidemiological studies have mainly used anthropometric variables such as WC and waist to hip ratio to estimate the proportion of abdominal adipose tissue. Adipose tissue is most simply categorized as subcutaneous adipose tissue (SAT) and visceral adipose tissue (VAT). SAT is mainly present in femoral-gluteal regions and on the back and anterior abdominal wall and accounts for around 80% of the body fat. VAT refers to the fat depots around abdominal viscera and accounts for up to 10-20% of total fat in men and 5-8% in women and the levels may increase with age [393]. Abdominal fat or central adiposity thus includes both SAT (anterior abdominal wall) and VAT (intra-abdominal) components.

WC is often used as a surrogate marker of central adiposity or abdominal adiposity in clinical and research settings. In a cross-sectional study including 3042 Korean men and women (1284 men, 362 premenopausal women, and 1396 postmenopausal women), men and post-menopausal women with highest WC and fat mass (assessed by DXA) had the lowest bone mass after controlling for weight [394]. In a similar study among Australian men and women (n = 1011) with a mean age of 63 ± 7 years and BMI of 28 ± 5 kg/m^2, WC and trunk fat (measured by DXA) was positively associated with higher vertebral deformities in women ($p < 0.05$). No consistent association was observed in men [387]. Consistent findings have been reported in the Nurses' Health Study consisting of 1168 women and 483 men who sustained a hip

fracture. After controlling for BMI, indicators of abdominal obesity (WC and waist-to-hip ratio) were associated with increased fracture risk in women only [395]. With every 10 cm increase in WC, there was a 13% higher risk of hip fracture (RR = 1.13; 95% CI 1.04-1.23) and with every 0.1 cm increase in waist-to-hip ratio, there was a 14% higher risk of hip fracture (RR =1.14, 95% CI 1.04-1.23) [395]. A meta-analyses of a large prospective cohort studies (n = 170,796) showed that WC was significantly associated with the increased risk of hip fracture in both men and women [392]. There was 55% higher risk of hip fracture in people with a WC of ≥ 105 cm as compared to those with a WC of <80 cm [392]. Further, there was a 3% (1.03 (95% CI 1.01–1.04)) higher risk of hip fracture with every 0.1 unit increase in waist: hip ratio [392].

1.10.4.2 Central Adiposity Measured as VAT and Fracture Risk

Studies using QCT, MRI or iDXA suggests that VAT is associated with poor bone microarchitecture and compromised strength [354, 355, 357, 396] while abdominal SAT may have no or even a protective effect [188, 396]. In the NTFS study, VAT was associated with prevalent vertebral fracture in women but not in men, despite no correlation between VAT and femoral neck or lumbar spine BMD, after controlling for body weight. Interestingly, even the major osteoporotic fracture risk (FRAX scores) did not differ between participants with and without vertebral fractures [188]. Bredella et al., in two separate cross-sectional studies have reported that higher abdominal VAT is associated with detrimental effect on bone health in young obese men [355] and premenopausal women [354]. Obese men (mean BMI = 36.5 ± 5.8 kg/m^2) with higher VAT measured by QCT had impaired bone microarchitecture and poor mechanical properties at the distal radius compared with obese men with lower amounts of VAT, despite similar BMI, lumbar spine and hip BMD determined by DXA. Abdominal VAT was negatively associated with Tb.Th (r = -0.5, p = 0.002), trabecular vBMD (r = -0.36, p = 0.04), failure load (r = -0.42, p = 0.01) and stiffness (r = -0.45, p = 0.008) at the distal radius [355]. Further, VAT accounted for 17% of the variability in failure load and for 20% of the variability in stiffness suggesting that VAT may be a significant contributor to detrimental effect of obesity on bone strength. There was a trend towards a positive correlation between abdominal SAT and Tb.N (r = 0.32, p = 0.08) and an inverse correlation with Tb.Sp (r = -0.32, p = 0.08). In sixty-eight obese premenopausal women

(mean BMI = 36.7 ± 4.2 kg/m^2), VAT was negatively associated with trabecular BMD independent of age and BMI (r = -0.42, p = 0.003) while no significant association between abdominal SAT and BMD was observed [354].

At the tissue level, compelling evidence is provided by a transiliac biopsy study in healthy premenopausal women (n = 40) which showed that women in the highest tertile of trunk fat as quantified by DXA (a surrogate for VAT) had inferior trabecular microarchitecture even though all subjects had normal BMD by DXA. The authors reported a strong correlation between trunk fat (measured by DXA) and VAT (r = 0.79, p < 0.0001) and not SAT (measured by CT) in an unselected subset of 34 people in this cohort [357]. Another study, examining 8833 CT scans of thorax or abdomen from 7230 patients between 18-64.9 years of age, showed that VAT was strongly and inversely associated with vertebral trabecular (r = -0.434; p < 0.001) and cortical (r = -0.407; p < 0.001) vBMD in both men and women independent of BMI and age [397].

Studies have pointed to the harmful effect of abdominal VAT even when BMI is within normal ranges. Gilsanz et al., in a cross-sectional study in 100 young (age range: 15 to 25 years) healthy females (between the 3rd and 97th percentile weight) reported that increased SAT (measured by QCT) was associated with higher cortical bone area, cross-sectional area, principal moments of inertia and polar moment of the femur bone (p < 0.05. for all) while VAT showed negative associations with the all these parameters (p < 0.05, for all) [396]. In a population based-prospective cohort study in elderly women, VAT was found to be associated with a 42% higher risk of non-spine fractures (OR: 1.42; 95 % CI, 1.09–1.85; p = 0.010) in women with normal BMI independent of age, ethnicity and BMD at the lumbar spine, femoral neck and total hip [398].

All these data suggest a detrimental effect of central adiposity on bone health are primarily driven by VAT depots independent of BMI and BMD. In contrast, in a longitudinal study higher VAT was associated with higher BMD and improved microarchitecture except for higher cortical porosity at the distal radius. However, when findings were adjusted for BMI, these associations were no longer significant, suggesting that the effect is mostly associated with obesity (higher mechanical loading) rather than with VAT [399].

1.10.4.3 VAT and BTMs

Several studies have reported that premenopausal women and young men with higher VAT have reduced levels of bone formation markers, resorption markers and reduced rate of turnover [354 , 355, 357]. In a cross-sectional study in premenopausal women (n = 68), bone formation marker (PINP) was shown to be positively correlated with SAT ($r = 0.25$, $p = 0.05$) and tended to be inversely associated with VAT ($r = -0.21$, $p = 0.09$) [354]. In another study in premenopausal women (n = 40), static and dynamic indices of bone formation such as wall width, bone formation rate and mineralizing surface were markedly lower in women with higher abdominal fat as compared to women with lower abdominal fat independent of age and BMI. Furthermore, bone formation rate was inversely associated with VAT measured by CT ($r = -0.37$, $p = 0.04$). Consistent with the histomorphometric data, serum levels of bone formation marker PINP was significantly lower for women with higher trunk fat (surrogate for VAT, $r = 0.79$, $p < 0.0001$). CTX-1 and TRAP5b were also lower suggesting an overall reduction in bone turnover rate. As well, serum levels of both resorption and formation markers were significantly and inversely associated with all measures of abdominal fat suggesting a detrimental effect of abdominal fat on bone remodelling. Serum CTX-1 levels were inversely associated with trunk fat ($r = -0.4$, $p = 0.011$), VAT ($r = -0.45$, $p = 0.012$) and SAT ($r = -0.36$, $p = 0.044$). Likewise, PINP was inversely associated with trunk fat ($r = -0.52$, $p < 0.001$), VAT ($r = -0.55$, $p = 0.001$) and SAT ($r = -0.55$, $p = 0.001$) [357]. The adverse association between VAT and bone observed in these studies provides further support to the notion that the distribution of fat in the body is an important predictor of poor bone health independent of BMI. However, these findings are limited to young women and whether such associations exist in post-menopausal women or elderly men need to be confirmed.

1.10.4.4 Hormonal Milieu Associated with VAT May Impact Bone Health

VAT is associated with increased levels of proinflammatory cytokines such as TNF-α, interleukins (especially IL-6), CRP which are known to increase bone resorption and promote osteoporosis as explained previously. In the NTFS cohort, women with higher incidence of vertebral fractures had significantly higher VAT and CRP levels [188]. Obesity is associated with lower adiponectin levels [400] and higher levels of leptin [401]

as compared to non-obese individuals. Adiponectin is an anti-inflammatory cytokine which suppresses TNF-α-induced NF-κB activation [402]. The actions of leptin on bone is complex and clinical studies have reported both positive [403, 404] and negative [405, 406] findings. Further, higher abdominal fat may be associated with higher marrow fat [353, 357]. Bredella et al [353] reported that premenopausal women (n = 47, mean BMI = 30 ± 7 kg/m^2) with higher VAT measured by QCT had higher vertebral bone marrow fat (measured using proton magnetic resonance spectroscopy) than those with lower VAT, independent of BMD (r = 0.34, p = 0.02). Another study reported higher iliac crest marrow fat in women with higher trunk fat (r = 0.37, p = 0.019) [357]. In contrast, Baum et al. [407] reported no significant association between VAT and vertebral bone marrow fat amongst 13 healthy post-menopausal women. Experimental evidence suggest that marrow adiposity is associated with reduced osteoblast number and enhanced osteoclastic activity [408].

1.10.5 Alterations in Calcium and Vitamin D metabolism in Obese Population

Several studies have reported alteration in calcium and vitamin D homeostasis in obese individuals, characterized by increased PTH levels [409-411] and reduced 25(OH)D levels [411-418]. With regards to circulating $1,25(OH)_2D$ levels, some studies have reported lower $1,25(OH)_2D$ levels [411] while others have reported elevated levels of $1,25(OH)_2D$ in obese people as compared to non-obese controls [412, 419].

i) Higher PTH: Significantly higher serum PTH levels have been reported among young and elderly obese men and women. PTH levels have been shown to be positively associated with body weight and fat mass even after adjustments for serum 25(OH)D levels [391, 409-411, 420]. Chronically elevated PTH levels may alter calcium metabolism and increase the proinflammatory cytokines levels and these may impact bone health negatively [421].

ii) Lower serum 25(OH)D levels: Many studies suggest an altered vitamin D endocrine system in obesity with significantly lower with lower 25(OH)D levels in obese men and women [412, 413, 417, 418, 422-425]. Several reasons for lower 25(OH)D levels in obese individuals have been postulated, including sequestration of vitamin D by adipose tissue

making it less bioavailable [417] or lower cutaneous synthesis with age [425]. However, most of the conclusive evidence suggests that greater volume of distribution in obese individuals results in lower 25(OH)D levels [418, 422]. Accordingly, the effect of dietary vitamin D supplementation have been found to be BMI dependent [426, 427] and hence it is recommended to adjust dietary vitamin D supplementation doses for body size to achieve optimal serum 25(OH)D levels and to suppress PTH levels. The Endocrine Society Clinical Practice Guideline also suggest 2–3 times higher vitamin D doses (respective to the age group) is required in obese people [428].

iii) Calcium requirements**:** Menopause is associated with reduced intestinal calcium absorption and increased calcium excretion with estrogen deficiency as discussed previously in section 1.5.1.1 and a higher intake is recommended to offset some of these biochemical & metabolic changes [116-119, 121]. Further, several reports have shown an inverse relationship between calcium intake and body fat mass [429-431]. Thus, obese postmenopausal women may benefit more by increasing their calcium intake-both in terms of reducing fat mass and protecting bone. An acute oral dose of calcium (500-1000 mg) in normal weight post-menopausal women, whether as a salt or as dietary calcium reduces CTX-1 levels and is associated with a concomitant reduction in PTH levels [50, 51, 66]. So far, none of the studies have evaluated the impact of calcium supplementation or dietary calcium intake on BTMs in overweight postmenopausal women. Also, it is not known if calcium intake needs to be adjusted to body weight to maintain bone health.

1.11 Implications of Obesity on bone health in Australian Population

The incidence of overweight and obesity worldwide is likely to increase significantly in the future including Australia. In fact, obesity is an important national issue with Australia's rate of overweight and obesity one of the highest in developed nations [432]. The latest data on the prevalence of obesity among Australians suggests that about two-thirds (67.0%) of Australian adults are overweight or obese (12.5 million people) [433]. The chances of morbidity and economic costs for treating fractures in overweight/obese individuals might be higher as compared to non-obese due to higher risk of fracture non-union and postoperative complications, comorbidities and slower rehabilitation [348]. Hence, preventive strategies for bone loss in overweight/obese individuals are of utmost clinical significance. Further, given that there is an alteration in calcium-vitamin D endocrine pathway with higher PTH levels and lower 25(OH)D, it is not clear if calcium intake should also be adjusted to body weight (as done for vitamin D) to maintain bone health and reduce fracture risk.

1.12 Aim 2 and Hypothesis

To the best of our knowledge, none of the studies so far have investigated bone remodelling in overweight healthy postmenopausal women and its association with VAT levels. Further, if obese premenopausal women remain in a suppressed state of bone resorption after menopause, it would be of importance to know how these women respond with acute changes in bone resorption markers post calcium supplementation. With an altered calcium metabolism and higher PTH levels, it would be helpful to ascertain which form of calcium supplementation will be more effective for women with higher VAT: dietary calcium or a calcium tablet.

The second phase of this PhD investigated associations between body composition variables i.e. VAT, SAT, lean mass with bone variables including whole body BMD, CTX-1 levels, PTH levels and vitamin D status in healthy postmenopausal women of varying BMI and VAT levels. Further, we investigated post prandial effects of two forms of calcium supplementation – dietary calcium (calcium from milk) and calcium tablet on CTX-1 levels and PTH levels.

1.12.1 Aim 2

Aim 2 a): To investigate the associations between body composition assessed by iDXA and metabolic markers of bone health in healthy post-menopausal women.

Aim 2b): To study acute effects of dietary calcium and a calcium supplement on CTX-1 levels in women with varying BMI and VAT levels.

We hypothesize that abdominal VAT will be negatively associated with all markers of bone health. Women with lower VAT will show a greater suppression in CTX-1 levels with both dietary calcium and calcium supplement as compared to women with higher VAT.

Chapter 2: Material and Methods

"Nothing has such power to broaden the mind as the ability to investigate systematically and truly all that comes under thy observation in life"

Marcus Aurelius

2.1 Structural and Biological determinants of Bone Health in Elderly

2.1.1 Participant Recruitment

Bone and serum specimens were retrieved from patients who presented to the Royal Adelaide Hospital (Adelaide, South Australia) between August 2015 and January 2018 for total hip replacement either for fractured neck of femur (fracture) or osteoarthritis (non-fracture). 111 fracture patients were recruited for the study. Of these, six patients were treated for osteoporosis (on anti-resorptive therapy) before fracture and two patients were taking calcitriol supplements; hence these eight patients were excluded from data analyses. Thus, the fracture cohort consisted of 103 patients, 65 females and 38 males with a mean age 85 ± 8.4 years. The non-fractured control group consisted of 25 patients (9 females, 17 males, aged 61.3 ± 13.5). During preoperative preparation for both non-fracture and fracture group, patients or next of kin were asked to participate in the study and written consent was obtained. The ethics approval for this study was obtained from Royal Adelaide Hospital Ethics Committee (Protocol no 130114; HREC/13/RAH/33) and University of South Australia Ethics Committee (HREC/35360).

2.1.2 Clinical Material and Records

Blood samples and intertrochanteric trabecular bone biopsies from the proximal femur and blood samples were collected by the surgeons at the time of surgery. Intertrochanteric trabecular bone biopsies were collected using two methods: initially a box chisel saw was used for the first thirty biopsies and subsequently a circular trephine was used to collect the remainder of the biopsies. Figure 2.1 shows a representative image of the bone core used in the study. All the samples were harvested from the intertrochanteric region of the femur and hence irrespective of the method of harvesting all specimens represented an anatomically consistent location. In the non-fracture group, the region of bone biopsied was distal to the sclerotic sub-chondral bone and hence considered appropriate for consideration as non-fracture control material.

A single blood specimen was collected at the time of surgery in serum separator tubes (SST) by the surgeons. Blood was centrifuged at 5000 rpm for 10 minutes within 2 hours of collection and the supernatant was collected in eppendorf tubes and stored at -80°C until further testing. Date of surgery, patient age, estimated glomerular filtration rate (eGFR), weight, height, comorbidities, medication history and total plasma levels of ionized calcium, total calcium, ionized calcium (estimated) [434], phosphate, albumin and total alkaline phosphatase were collected from patient case reports.

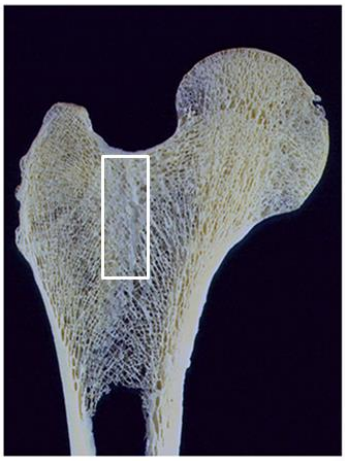

Figure 2. 1 Region of bone specimen retrieved for analyses

2.1.3 Biochemical Analyses

Serum samples were analyzed by chemiluminescent immunoassay (Diasorin LiaisonXL) for 25(OH)D levels and 1,25$(OH)_2$D levels, 1-84 PTH levels (3rd generation assay; 1-84 PTH), intact PTH levels (7-84 Parathyroid hormone, 2nd generation assay; iPTH), osteocalcin and bone ALP. 400µl of specimen was added to the sample tubes. The volume was calculated based on sample required for each of the assay and the dead volume needed for the instrument. The calibration for each of the assay was performed after equipment and reagent stabilization. Following this, quality controls level 1 and level 2 were run. Once quality control results indicated appropriate performance for each assay, patient samples were loaded on the Liaison equipment and tests were selected. The principle for each of test is detailed below.

2.1.3.1 Calcifediol (25(OH)D) Assay

The LIAISON® 25(OH)D assay is a direct competitive chemiluminescence immunoassay (CLIA) for quantitative determination of total 25(OH) levels in serum. During the first incubation, 25(OH)D is dissociated from its binding protein and binds to the specific antibody on the solid phase. After 10 minutes the tracer, (vitamin D linked to an isoluminol derivative) is added. After a second 10-minute incubation, the unbound material is removed with a wash cycle. Subsequently, the starter reagents are added to initiate a flash chemiluminescent reaction. The light signal is measured by a photomultiplier as relative light units (RLU) and is inversely proportional to the concentration of 25(OH) levels present in the specimen samples. Values were reported in nmol/L. The precision CVs as provided by the manufacturer at concentrations of 19.6 and 301.3 nmol/L are 3.8% and 0.1% (within run) and 9.8% and 9.1% (between run).

2.1.3.2 Calcitriol (1,25(OH)$_2$D) Assay

The LIAISON® 1,25 (OH)$_2$ D assay is a modified 3 step sandwich assay that uses a recombinant fusion protein for capture of the 1,25(OH)2D molecule and a murine monoclonal antibody which specifically recognizes the complex formed by the recombinant fusion protein with the 1,25(OH)$_2$D molecule. During the first incubation, test specimens are incubated with the recombinant protein (binding agent) and assay buffer. Following this incubation, the solid phase containing the specific monoclonal antibody is added and allowed to bind with the complex from the first incubation. After the second incubation, a wash cycle is performed to remove any unbound material. The third step consists of adding the conjugate and incubating. The unbound material is removed with a second wash step. The starter reagents are then added, and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as RLU and is proportional to the concentration of 1,25(OH)$_2$ D levels present in the sample. Values were reported in pmol/L. The precision CVs as provided by the manufacturer at concentrations of 30.9 and 122.9 pmol/L are 2.7% and 5% (within run) and 3.8% and 3.6% (between run).

2.1.3.3 Bone ALP Assay

The Liaison® bone ALP immunoassay is a direct two-site sandwich assay that utilizes two affinity purified mouse monoclonal antibodies for capture and detection of BAP. The serum sample is incubated with paramagnetic particles coated with a BAP monoclonal antibody. Following incubation, a second isoluminol conjugated monoclonal antibody is added to the reaction for a short incubation. After the incubation, the unbound material is removed with a wash cycle. The starter reagents are then added, and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as RLU and is proportional to the concentration of bone ALP levels present in the samples. Values were reported in nmol/L. The precision CVs as provided by the manufacturer at concentrations of 6.83 and 37.9 µg/L are 3.4% and 1.9% (within run) and 3.9% and 3.3% (between run).

2.1.3.4 Osteocalcin Assay

The method for quantitative determination of osteocalcin is a direct, 2 site, sandwich type CLIA. Affinity-purified mouse antibody to synthetic human osteocalcin is coated to the solid phase. The second affinity-purified mouse antibody is conjugated to an isoluminol derivative. During the incubation, osteocalcin binds to the solid phase and is subsequently bound by isoluminol conjugated antibody. After the incubation, the unbound material is removed with a wash cycle. The starter reagents are then added, and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as RLU and is proportional to the concentration of osteocalcin levels present in sample. Values were reported in nmol/L. The precision CVs as provided by the manufacturer at concentrations of 10.4 and 238 ng/ml are 12% and 4% (within run) and 14% and 6% (between run).

2.1.3.5 1-84 PTH assay

The LIAISON® 1-84 PTH assay is a modified two-step, two-site sandwich assay that uses two polyclonal antibodies for capture and detection of the 1-84 PTH molecule. Human serum is incubated with an isoluminol conjugated polyclonal antibody with high specificity

for the N-terminus of the 1-84 peptide. Following incubation, paramagnetic particles coated with a second polyclonal antibody that binds to the C-terminal region of the 1-84 molecule are added to the reaction and incubated. The use of these antibodies guarantees that only 1-84 PTH is detected with no cross reactivity to fragments such as the 7-84 PTH fragment. After the second incubation, the unbound material is removed with a wash cycle. The starter reagents are then added, and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as RLU and is proportional to the concentration of 1-84 PTH levels present in the sample. Values were reported in pmol/L. The precision CVs as provided by the manufacturer at concentrations of 3 and 30.5 pmol/L are 4.9% and 4.8% (within run) and 7.2% and 5.6% (between run).

2.1.3.6 Intact PTH (iPTH) assay

The LIAISON® iPTH Gen II assay is a modified 2-step, 2-site sandwich assay that uses 2 polyclonal antibodies for capture and detection of intact PTH. Patient sample is incubated with an assay buffer and an isoluminol conjugated polyclonal affinity purified antibody to the 1-34 region of the 1-84 PTH molecule. Following incubation, paramagnetic particles coated with a second polyclonal antibody with a tendency to bind in the C terminal region (39-84) of the 1-84 PTH molecule are added to the reaction and incubated. After the second incubation, the unbound material is removed with a wash cycle. The starter reagents are then added, and a flash chemiluminescent reaction is initiated. The light signal is measured by a photomultiplier as RLU and is proportional to the concentration of iPTH present in the sample. Values were reported in pmol/L. The precision CVs as provided by the manufacturer at concentrations of 2 and 26.5 pmol/L are 1.3% and 3.7% (within run) and 3.3% and 5.5% (between run).

2.1.4 Bone biopsy preparation

The trabecular bone biopsies were formalin fixed (10% formalin) for 7 days at 4 °C, followed by submersion in 70% ethanol at room temperature until micro CT and histological analyses could be performed. A portion of bone was also submerged in RNA-Later™ (Ambion, Warrington, UK) and stored as per manufacturer's instruction at -80°C for

gene expression analyses.

2.1.5 Bone biopsy Micro-computed tomography (micro-CT)

Formalin-prepared trabecular bone specimens were assessed for bone mass and bone quality variables using the 1076 Bruker micro-CT imaging system. Samples were packed appropriately in specimen container with 1-2ml of 70% ethanol to prevent dehydration. For image acquisition, the X-ray tube was operated at 60KV and 167µA with a resolution of 18µm with a 0.5mm aluminum filter and exposure time of 1770ms. Camera rotation was set at 0.6. After the scanner calibration, a flat field was run each time before starting specimen scanning to obtain a uniform image background. The images were reconstructed using the CTAn software (v1.13, Kontich, Belgium) after correcting for smoothing (1), beam hardening (30%) and maximum ring artifact reduction (20). The mean specimen volume scanned was 1093.7± 511.2 mm^3 with a range of 291.7 to 1078.8 mm^3. Morphometric indices were determined using an adaptive thresholding algorithm with a pre-thresholding of 60 to 255 and a radius of 8. A detailed description of the variables measured is given in chapter 1 (section 1.5.2.2.1 and table 1.4).

Briefly, bone quantity variables included BV/TV, Tb.N and Tb.Sp and trabecular bone quality measures included BS/BV, Tb.Th, Tb.Pf and SMI. The measurement of BS/BV provides an integrated measure of thickness and texture of the bone. BS/BV is inversely related to Tb.th i.e. a thicker bone with a higher number of trabecular plates will have a lower bone surface to bone volume ratio. Tb.Pf is an indicator of the surface curvature. A well-connected trabecular bone has concave curvature indicating higher connectivity with lower Tb.Pf values. Convex curvature is characteristic of disconnected structures which increases Tb.Pf. SMI is an index of the ratio of plates to rods. The values of SMI range between 0 and 3, with 0 indicative of structures with only plates and 3 indicative of a structure consisting of rods only.

2.1.6 Bone Biopsy Histology

Formalin-fixed intertrochanteric trabecular bone biopsies were processed through

70%-100% ethanol to dehydrate the sample and then placed in 5ml solution containing 100% methylmethacrylate (MMA) and 10% v/v polyethylene glycol (PEG) and stored at room temperature for 10-14 days. Resin embedding then occurred using a solution of 100% MMA, 10% PEG and 0.4% peroxydicarbonate. Biopsies were submerged in 5ml of this solution and placed in at 37°C for 24 hours to allow the resin to harden. A volume of 5 µm thick decalcified sections were cut and stained by Haematoxylin and Eosin [435]. Mean wall thickness (MWT), a measure of completed packets of bone remodelling, were measured in each section (figure 2.2 A-B, adapted from [168]). Wall thickness was defined as a complete trabecular bone packet on straight, rod-like trabecular structures. Five sites (10 measurements per site) per section were obtained between perpendicular cement lines beneath bone where no resorption of osteoid formation occurred.

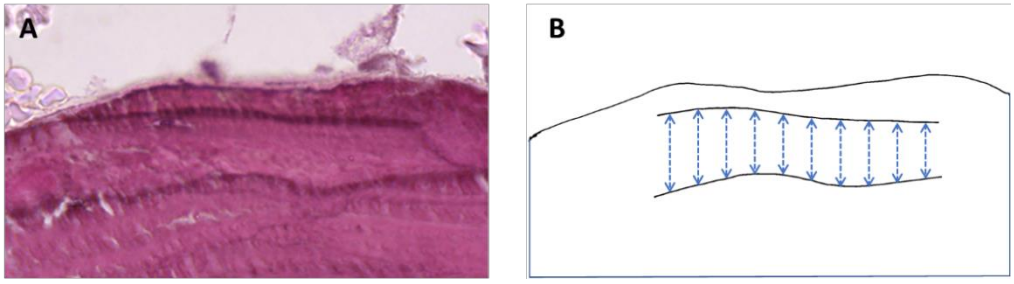

Figure 2. 2A. Representative histological section indicating packet of trabecular bone and b. representative measurements between cement lines

2.1.7 Bone mRNA Analyses

Total RNA was extracted from the pulverized bone using the TRIZOL reagent (Life-technologies), and cDNA samples were synthesized (Superscript-III kit, Life Technologies). The mRNA expression of genes of interest was measured by real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), as per the manufacturer's instructions. Relative gene expression was quantified delta-CT method and normalizing to the expression of β-actin mRNA. The primer sequence for CYP27B1 is TGGCCCAGATCCTAACACATTT (forward) GTCCGGGTCTTGGGTCTAACT (reverse) CYP24A1 CCTGCTGCCAGATTCTCTGGAA (forward) TTGCCATACTTCTTGTGGTACTCC (reverse) and

Beta actin CTGCCCTGAGGCACTCTT (forward) AGTTTCGTGGATGCCACAGG (reverse).

2.1.8 Radiographic Cortical Bone Analyses

AP plain radiographs of the contralateral side were used to measure average cortical thickness, medullary width and total femoral width using CARESTREAM software. A description of these variables is shown in figure 2.3. Average cortical thickness is measured by average of region 1 and 2. Medullary width and total femoral width is represented by region 3 and 4, respectively. Each region was measured three times for every patient and the CV was between 2 and 5%.

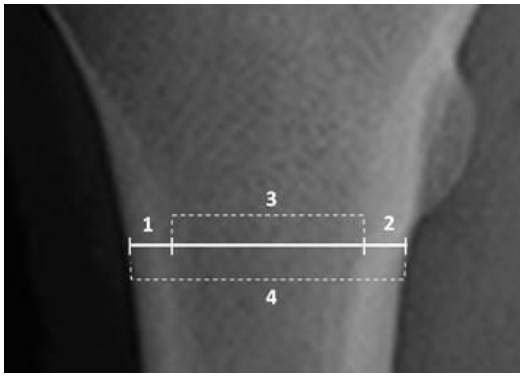

Figure 2. 3 Radiograph for cortical bone measurements

2.1.9 Statistical Analyses

Statistical analysis was performed using the SPSS statistical Package (IBM SPSS Stats 25, St Leonards, Australia). Some of the variables were not normally distributed, hence values are expressed as median and interquartile range. Mann-Whitney U test was used to compare the difference in structural parameters between hip fracture patients and non-fracture controls. Spearman's correlation coefficient was calculated to determine correlations between structural, metabolic and gene expression variables. The metabolic variables which correlated with structural variables were tested through step-wise multiple linear regression analyses to identify metabolic determinants of bone mass and bone quality variables. Independent samples Kruskal–Wallis test was used to compare bone structural and quality variables between various 25(OH)D intervals. The significance level

was set at 0.05.

2.2 Effect of Visceral Fat Levels on BTMs and Response to Acute Calcium supplementation

2.2.1 Participant Recruitment

Advertisements (figure 2.4) were placed in the free community newspaper Seniors (August 2017) and Messenger (November 2017) circulated in the Northern and North Eastern suburbs of Adelaide, encouraging postmenopausal women to participate in a study investigating the acute effects of two different forms of calcium supplementation on a bone resorption marker in lean and overweight women. In addition, messages were added to coast FM and Facebook between the months of July- September 2017 (figure 2.4).

All interested women were asked to contact University of South Australia clinical trial facility by telephone or email. Upon receipt of email or phone call, interested volunteers were sent the participant information sheet (Appendix I) which had complete details of the study, inclusion and exclusion criterions and risk involved in participating. Volunteers who expressed interest for participation were invited to the clinical trial facility for a screening visit to assess their suitability for the study. The participants who met the BMI criterion and with other inclusion criteria listed in table 2.1 were explained the study in detail and informed consent was obtained (Appendix II). The ethics approval for this study was obtained from University of South Australia Human Ethics committee, protocol no 36365 and the trial was registered at Australia New Zealand Clinical Trial Registry (ANZCTR) as ACTRN12617000779370.



Figure 2. 4 Advertisement as it appeared in the messenger community newspaper and the Facebook ad

Table 2. 1 Inclusion and exclusion criterion for the clinical study

Inclusion Criteria
• Postmenopausal women (more than 5 years since menopause). • Non-diabetic and lactose-tolerant. • Women who have never been on antiresorptive treatment. • BMI less than 24.9kg/m^2 or greater than 27 kg/m^2 • Not taking calcium or vitamin D supplement, or, if so, agreeing to stop these for 4 weeks before entering the trial.
Exclusion Criteria
• Women with BMI in the range of 25-26.9 kg/m^2. This criterion was set to clearly differentiate between the effect of obesity (VAT) on suppression of bone resorption marker with different calcium treatments. • Treatment with bisphosphonates (any dose within the previous 2 years) or other medications known to affect bone (within the previous 6 months). • Participant's medical history was recorded to check for ongoing conditions known to cause abnormalities of calcium metabolism or skeletal health such as osteoporosis, malabsorption syndromes (such as celiac or crohn's disease), hyperthyroidism, diabetes, hyperparathyroidism, osteomalacia, fracture in the past 12 months, known chronic kidney disease, participation in any clinical trial of active therapy within 3 months pre-randomization. Such conditions are known to affect bone turnover and to minimize confounding factors, these participants were excluded from the study.

2.2.2 Screening Visit

2.2.2.1 Anthropometric Assessment

During the screening visit, after signing the consent form, participants were requested to complete an information sheet that provided details on medication, gynecological history and other information (Appendix III). All participants underwent body

weight measurements to the nearest 0.1 kg using standardized scales and standing height to the nearest 0.1 cm using a stadiometer. Hip and waist WC were recorded to the nearest 0.1 cm using a non-stretchable measuring tape. WC was measured at midpoint between the top of the iliac crest and the lower margin of the last rib [436]. Hip circumference was measured at the largest circumference of the buttocks [436]. Total body DXA scans were acquired using the lunar iDXA (GE Healthcare, Madison, WI) to measure whole-body fat mass, lean mass, android fat, gynoid fat, A:G ratio, total body BMD. CoreScan software was used to measure SAT and VAT over the android region. The android region is roughly 10 cm in height, extending from the iliac crest towards the head a distance that is 20% of the distance from the iliac crest to the base of the mandible (figure 2.5). CoreScan software automatically defines the android region on each participant to avoid any inter- or intra-observer variance. The distinction between VAT and SAT layers are made using geometric calculations and attenuation measurements automatically by the machine. The CoreScan algorithm works through detection of two key parameters: the width of the SAT layer on the lateral aspects of the abdomen (figure 2.5) and the anterior-posterior thickness of the abdomen (figure 2.5), which can be attained using the DXA tissue attenuation image. a simple geometric model using these measures is used to estimate the android SAT. VAT is computed by subtracting the android SAT from the android fat. CoreScan algorithm has been validated using paired DXA and volumetric CT scan images from 124 male and female participants. The population group included men and women over a wide range of age (18-90 years) and BMI (18.5-40 kg/m^2) [437, 438]. CoreScan software automatically defines the android region on each participant to avoid any inter- or intra-observer variance. The distinction between VAT and SAT is made using geometric calculations and attenuation measurements automatically by the machine. All measurements were recorded in a de-identified case report form (CRF) for every participant (Appendix IV). Hundred and twelve participants volunteered to take part in the study and attended the screening visit. Out of the 112 participants, 14 had BMI between 25-26 kg/m^2 and were excluded. Ninety-eight participants were enrolled and underwent DXA assessment. Of these, two participants informed later that they had been on hormone replacement therapy in the past and were excluded, one was an epileptic patient and withdrew, one had sustained a fracture in the

past 12 months and was excluded. Thirteen participants expressed issues with cannula insertion and withdrew after the screening visit. Eighty-one participants attended the second visit, of which four participants had tricky veins and wished to withdraw from the trial. Hence, 77 participants completed the intervention visits (2,3 and 4).

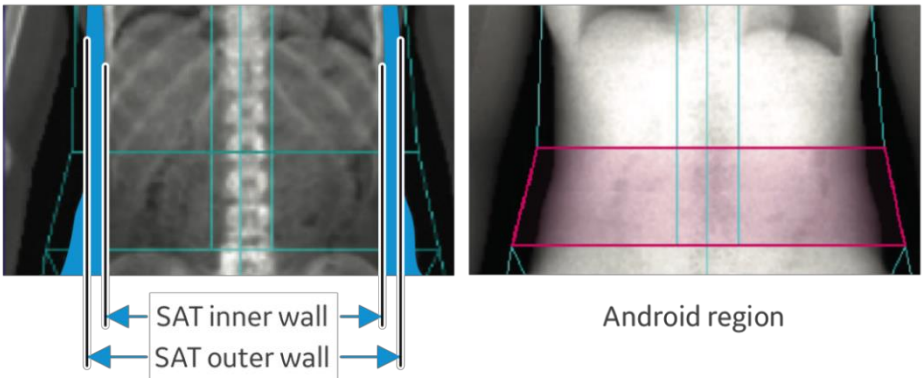

Figure 2. 5 The android region over the abdomen of a total body iDXA scan

2.2.2.2 Treatment Allocation

Each participant was then allocated a study number and was randomized to a sequential treatment plan to consume each of the test products. The treatment allocation plan was generated using a random code generator before study commencement. The three test products were:

- Calcium supplement with water – 1000 mg calcium supplement with 500 mL water (Brand name Calsup)
- High calcium milk - providing 1000 mg calcium per 500 ml (Brand name: LIDDELLS lactose free HI CALCIUM SKIM MILK)
- Non fortified orange fruit juice- 500 ml, no calcium.

The nutritional composition of high calcium milk and juice is given in table 2.2. The participants were booked in for three consecutive weeks depending on their convenience to start the intervention. The prerequisites such as fasting for 10 hours prior to the visit, to cease any calcium supplement one-month prior were explained to the participants.

Table 2. 2 Nutritional composition for high calcium milk and orange juice

Nutrient	High Calcium Milk (per 500ml)	Orange Juice (per 500ml)
Energy (kcal)	210	210
Protein (g)	19.5	Nil
Fat (g)	0.5	Nil
Carbohydrate (g)	31.5	52.5
Calcium (mg)	1000	Nil
Vitamin D (µg)	2.5	Nil

2.2.3 Intervention Visits

All the participants returned to the clinical trial facility after one or two weeks depending on their availability and commitment for three weeks in continuation. The participants arrived at the laboratory between 8.00-8.30am after an overnight fast. Upon arrival, an indwelling intravenous cannula was placed in an antecubital vein and kept patent using saline. Baseline serum and plasma specimens were collected shortly after placing the canula. The participants were then given their allocated product and were requested to consume the same within 15 minutes. Time zero was recorded as the time when the participant started drinking the product. Subsequent serum and plasma specimens were collected at 30 minutes, 1, 2, 3, 4 and 5 hours. The blood specimens were stored on ice and centrifuged at 4000 rpm for 15 minutes within 2 hours of collection. The samples were stored in multiple aliquots at -80^{0}C till further analyses. The participants returned to the laboratory twice after a wash out period of one week each and the same procedure as described above was repeated with a change in the intervention depending on the participant allocation. Hence, all participants were administered all the three interventions i.e. high calcium milk, calcium carbonate tablet and orange juice. All the study visits were documented in individual case report forms (Appendix IV). The details of all the study visits is summarized in figure 2.6.

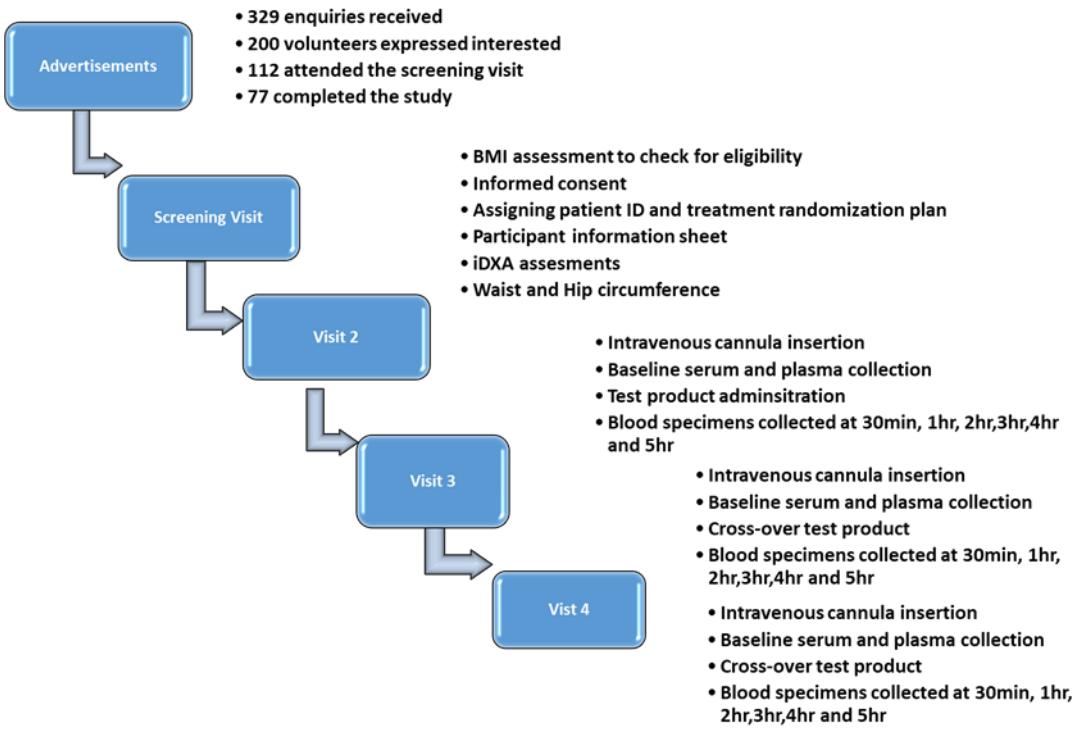

Figure 2. 6Flowchart showing details of the four study visits

2.2.4 Biochemical analyses

2.2.4.1 Plasma CTX-1 levels measurements

CTX-1 was measured using fully automatic IDS-iSYS immunodiagnostic system at CTX-1 was measured at baseline, 30 minutes, 1-hour, 2-hour, 3-hour, 4-hour and 5-hour time points. After machine and reagent stabilization, calibration and quality control (QC) were performed. Calibration was repeated after 15 days whereas QC's were performed daily before starting specimen measurements and with every new reagent kit. The analyses were performed over a period of month. The precision CVs as provided by the manufacturer at a concentrations of 216 ng/L, 877 ng/L and 2270 ng/L are 4.9%, 2.1% and 2.4% respectively (within run) and 8.8%, 5.1% and 4.7 % respectively (between run). The percent CV for CTX-1 measurement in our laboratory was 5% (figure 2.9). CTX-1 levels were measured using 2 step chemiluminescence immunoassay. Participant plasma sample was incubated with two highly specific monoclonal antibodies against different epitopes of the amino acid sequence EKAHD-β-GGR. One is conjugated to biotin and the other to acridinium. Streptavidin-coated magnetic particles are then added and incubated. The magnetic particles are captured using a magnet and a wash step is performed to remove any unbound analyte. Trigger reagents are added; the resulting light emitted by the acridinium label is directly proportional to the concentration of analyte in the sample. The reportable range of the assay is 33-6000 ng/L. Any value that read below 33 ng/L will be reported as < 33 ng/L.

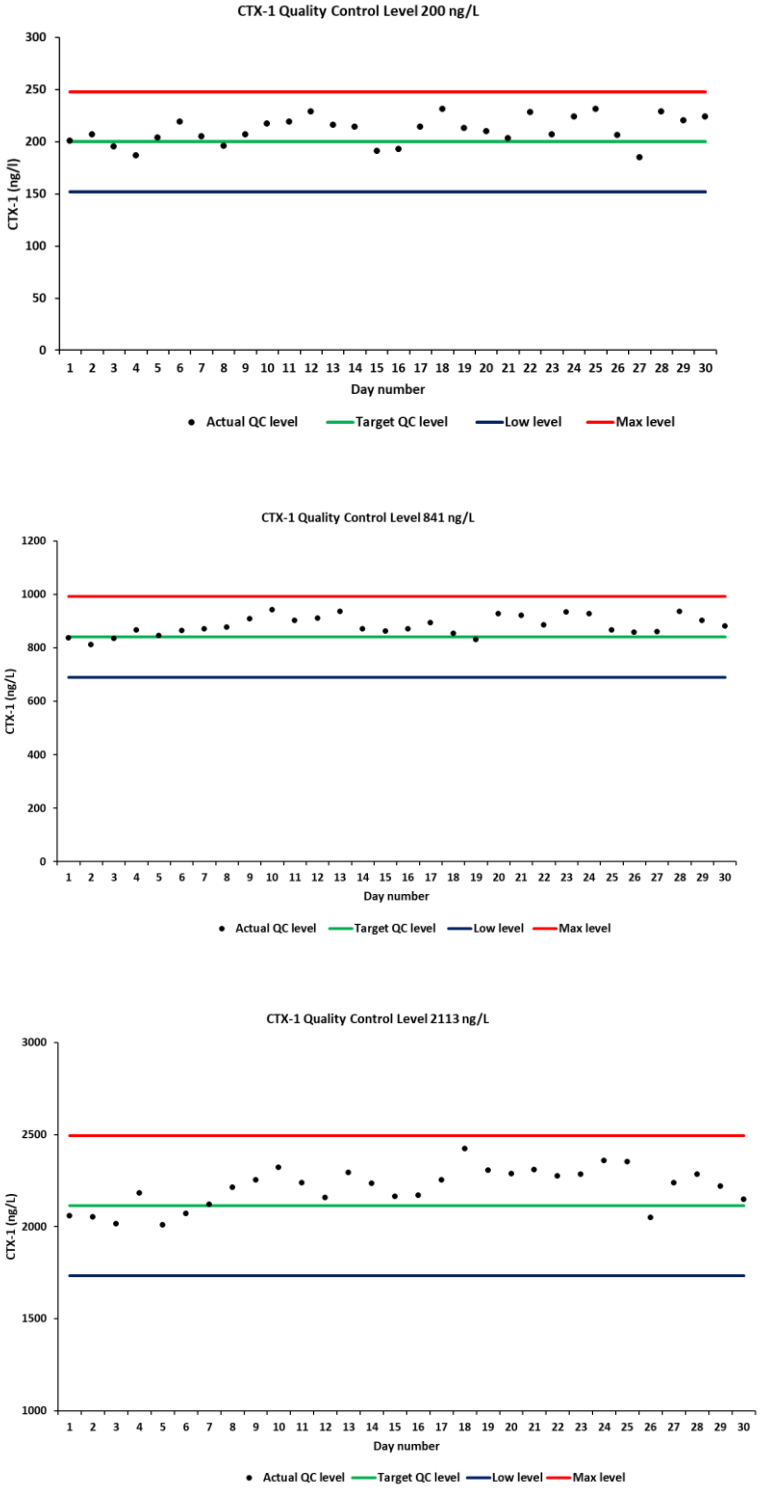

Figure 2. 7 Quality control data for CTX-1 analyses

2.2.4.2 PTH Measurements

1-84 PTH and iPTH levels were measured by Liaison two-step, two-site sandwich assay as described above in 2.1.3.5 and 2.1.3.6. 1-84 PTH levels were measured at baseline, 1-hour, 2-hour, 3-hour, 4 hour and 5-hour time point. iPTH was measured at baseline and 1-hour timepoints only to assess the correlation between iPTH and 1-84 PTH assay.

2.2.4.3 Calcium, Phosphorus and ALP Measurement

The serum samples were analyzed for total calcium, phosphorus and alkaline phosphatase using an automated Thermo Fisher Scientific automated Kone lab analyzer. All the three analytes were measured simultaneously on the Kone machine. After machine and reagent stabilization, calibration and QC's were performed. ALP was calibrated against eCal, code 981830 and calcium & phosphorus were calibrated against sCal, code 981831 as per manufacturer's instructions. The quality control materials Abtrol, code 981044 and Notrol, code 981043 were run to ensure accuracy of results. The accuracy of the data was also verified using Biorad Liquichek™ Unassayed chemistry control (human) levels 1 and 2. The percent CV for calcium, phosphorus and ALP was 1.6-5.8%, 0.6-1.8% and 3.6-9.5%, respectively. After performing calibration and QC, 200µl of serum samples was added to the sample cups and loaded on to the machine. The assays were run as per manufacturer instructions. Calcium and phosphorus values were expressed in mmol/L, ALP in U/L. Calcium and phosphorus were measured at baseline, 1- hour, 2-hour, 3-hour, 4 hour and 5-hour time points. ALP was measured only at baseline, 2-hour and 5-hour time points. The principle behind measurement of each analyte is described below.

Total serum calcium was estimated using automated Konelab analyzer. Calcium ions form a colored complex with Arsenazo III at neutral pH. The amount of complex is measured at 660nm. The results are calculated automatically by the analyzer using a calibration curve.

ALP catalyzes the hydrolysis of p-nitrophenylphosphate to form phosphate and p-nitrophenol. The formation of p-nitrophenol in alkaline solution is measured at 405 nm. Results are calculated automatically by the analyzer using a calibration curve.

Phosphate forms in acidic medium a phosphomolybdate complex with ammonium molybdate. The absorbance at 340 nm is directly proportional to the concentration of inorganic phosphate in the sample. Results are calculated automatically by the analyzer using a calibration curve.

2.2.4.4 Other Measurements at Baseline (Timepoint 0)

Baseline 25(OH)D levels, 1,25$(OH)_2$D levels, osteocalcin levels and bone ALP levels were measured as described above in section 2.1.3 using Liaison immunoassay.

2.2.5 Statistical Analyses

Normality was assessed with the use of histograms and Shapiro-Wilk test. Most of the variables (BMD, BMI, WC, CTX-1 levels, bone ALP levels, 1,25$(OH)_2$D levels, 1-84 PTH levels) were not normally distributed, hence Spearman's coefficient of correlation was used to describe the relationships between body fat variables and bone markers. To investigate the effect of VAT on bone markers and total body BMD, participants were divided into VAT quartiles and non-parametric Kruskal-Wallis test was used to compare the quartiles. Multiple linear regression models were used to investigate determinants of bone and biochemical variables. CTX-1 and 1-84 PTH values were log transformed at all time points and repeated measures of ANOVA was used to investigate the interaction between VAT, calcium intervention and timepoints using VAT quartiles as a-between subject factor. The mean maximal suppression in CTX-1 levels were calculated and expressed as percent change from the baseline value for each of the test product. All the analyses were carried out using SPSS (IBM SPSS Stats 25, St Leonards, Australia) and $p < 0.05$ was considered significant.

Chapter 3: Structural and Metabolic Profile of Fracture Patients – Association of Vitamin D with Bone Quality and Bone Quantity Measures

"Your assumptions are your windows on the world. Scrub them off every once in a while, or the light won't come in."

Isaac Asimov

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Elevated Serum 25-Hydroxyvitamin D Levels Are Associated with Improved Bone Formation and Micro-Structural Measures in Elderly Hip Fracture Patients. J Clin Med, 2019. ***8****(11).*

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Conceptualization, D.K.S., P.M.C., H.A.M. and P.H.A.; methodology, D.K.S., R.K.S., R.S., P.M.C., H.M.A. and P.H.A.; statistical analysis, D.K.S., R.K.S., R.S., P.M.C., H.A.M. and P.H.A.; resources, T.S.R., L.B.S., G.J.A., H.A.M. and P.H.A.; data curation, D.K.S., P.M.C., H.A.M. and P.H.A.; writing—original draft preparation, D.K.S. and P.H.A.; writing—review and editing, D.K.S., T.S.R., R.S., P.C.M. and P.H.A.; visualization, D.K.S., R.K.S., P.M.C. and P.H.A.; supervision, L.B.S., G.J.A., P.H.C., H.A.M. and P.H.A.; project administration, H.A.M. and P.H.A.; funding acquisition, P.M.C., H.A.M. and P.H.A.

All authors reviewed and approved the final manuscript.

STATEMENT OF AUTHORSHIP

Elevated Serum 25-Hydroxyvitamin D Levels Are Associated with Improved Bone Formation and Micro-Structural Measures in Elderly Hip Fracture Patients. J Clin Med, 2019. 8(11).

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I hereby certify that this statement of contribution to the above-named paper is correct.
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5/04/2020

Author 2:
Rebecca Sawyer
Ms Rebecca Sawyer was responsible for performing the histological analyses and approving the final manuscript.
I hereby certify that this statement of contribution to the above-named paper is correct.
Signed:

6/04/2020

Author 3:
Thomas Robertson
Dr Thomas Robertson was responsible for providing patient information including age, date of birth, date of surgery, comorbidities and baseline medication and supplement intake data.
I hereby certify that this statement of contribution to the above-named paper is correct.

Author 4:
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Dr Roumen Stamenkov was responsible for performing cortical bone analyses and provided the cortical radiograph for visualization
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Author 7:
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Signed:

6th April 2020
Author 8:
Howard A Morris (deceased)
Prof Howard Morris conceptualized this research, supported with data analyses and interpretation and manuscript reviewing and editing and provided funding and all the resources for this work.
I hereby certify that this statement of contribution to the above-named paper is correct.
Signed:
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Author 9:
Paul H Anderson
Prof Paul H Anderson conceptualized this research, provided guidance and supervision for all laboratory work, data analyses and interpretation, manuscript reviewing, editing, approving the final manuscript.
I hereby certify that this statement of contribution to the above-named paper is correct.
Signed:

6/04/2020

3.1 Introduction

The strength of a bone is determined by quantity, quality and microarchitecture as discussed in chapter 1 (section 1.5.2). Studies have shown that patients who suffer from an osteoporotic fracture and those who do not, have a large overlap in BMD [180], suggesting that while low BMD is a strong risk factor for fractures, using only a T-score BMD value below -2.5 as an intervention threshold may not sufficiently identify at-risk population for fragility fracture [181].

Micro-CT can be used to quantify the microarchitecture of trabecular bone [439]. The evaluation of three-dimensional morphology of trabecular bone provides an estimation of several indices, such as BV/TV, Tb.Th, Tb.Sp, Tb.N as well as information about the trabecular plate or rod-like shape. These indices have been used to examine human trabecular bone in different anatomical locations such as vertebrae, iliac crest, proximal femur and calcaneal bone [440-442]. In this chapter, we first report a comparison of trabecular bone microarchitecture in fracture and non-fracture patients focusing on measurements of bone mass and bone quality including Tb.Th, texture prevalence of trabecular plates and rods as well as connectivity of individual trabeculae. These data provide further insights on the microarchitectural changes associated with fragility fractures.

We then describe the association of vitamin D with trabecular bone quality, quantity and cortical bone in fracture patients. Lower serum 25(OH)D levels, have been shown to be associated with increased risk of hip fracture in the elderly [443]. Despite this knowledge, questions remain as to mechanisms by which higher 25(OH)D levels improve skeletal health [18], especially since levels above 20 nmol/L are considered adequate for renal $1,25(OH)_2D$ synthesis and for intestinal calcium absorption [303, 305]. RCTs involving vitamin D supplementation and assessing BMD as an outcome have shown minimal effect of vitamin D. It may well be possible that vitamin D affects bone health through mechanisms independent of BMD. This point is further elucidated by comparing the results

of studies that have investigated the effect of vitamin D on different bone outcomes. A large RCT VIDA (n = 5110, aged 50-84 years) recently demonstrated that monthly doses of vitamin D3 (100,000 IU) for 3.4 years resulted in no reduction in fracture risk [316]. Results of a sub study of the ViDA trial involving 452 community-dwelling older adults (mean age 69 years) investigating the effects of this monthly vitamin D supplementation on BMD suggest no clinically important benefit on bone mass. The benefit was only reported in participants with baseline serum 25(OH)D levels less than 30 nmol/L [36]. In contrast, studies that have investigated effect of vitamin D supplementation on bone microarchitecture have shown significantly higher Tb.Th [444], altered mineral properties [445] and improved bone strength [444]. Hence, to gain greater insights on the role of vitamin D on bone quality and microarchitecture, we investigated the association of low and high vitamin D status on bone microarchitecture using high resolution 3D micro-CT system.

To accomplish these aims, a large cohort of patients presenting at RAH for hip arthroplasty due to fractured neck of the femur or elective hip arthroplasty were recruited. Blood and intertrochanteric trabecular bone biopsies were collected by the surgeons at the time of surgery. The microarchitectural and metabolic differences were compared between the fracture and the non-fracture group. The effect of serum 25(OH)D levels, $1,25(OH)_2D$ levels and PTH levels on bone microarchitecture and gene expression was investigated in the fracture cohort. The decision for selecting the fracture cohort to study the association between vitamin D status and bone quality was based on the findings of our pilot study which investigated the feasibility of conducting the present cross-sectional study. The pilot data indicated that in 50% of the hip fracture patients, serum 25(OH)D levels are less than 50 nmol/L and in 25% greater than 75 nmol/L providing a distribution of levels associated with the loss and maintenance of bone. We estimated that at least 100 patients would be required to demonstrate clinically relevant differences in bone microarchitecture in patients with varying 25(OH)D levels. Since the rate at which hip fracture patients presented at RAH was significantly higher than the patients presenting for elective hip arthroplasty, we decided to study the association of vitamin D status in the fracture cohort.

The intertrochanteric region is distant from the fracture site in the fracture patients and unlikely to be affected by disease in non-fracture hip osteoarthritis patients. Hence, we believe it is a relevant anatomic position to study the association between vitamin D levels and trabecular bone microarchitecture. For investigating the effect of vitamin D levels on cortical bone, radiographs from the contra-lateral side were used.

3.2 Materials and Methods

3.2.1 Participant Recruitment and Clinical Material

Elderly hip patients as described previously in section 2.1.1 were recruited for the study. Briefly, patients presenting for hip arthroplasty due to fractured neck of femur or elective hip arthroplasty due to osteoarthritis of the hip at RAH were recruited for the study. Blood and intertrochanteric trabecular bone biopsies were collected from 103 patients fracture patients (65 females, 38 males; aged 85 ± 8.4 years) and 27 non-fracture hip arthroplasty patients (10 females and 17 males, aged 61.3 ± 13.5) as described in section 2.1.2. Patient records were collected to determine age, date of surgery, eGFR, weight, height, comorbidities, medication history and plasma levels of ionized calcium, total calcium and albumin. All these parameters were determined and recorded prior to the surgery. The ethics approval for this study was obtained from Royal Adelaide Hospital Ethics Committee (Protocol no 130114; HREC/13/RAH/33).

3.2.2 Sample Preparation

The trabecular bone biopsies were processed for 3D micro CT structural analyses and gene expression as described in section 2.1.4.

3.2.3 Biochemical Analyses

Serum samples were analyzed for 25(OH)D (section 2.1.3.1), 1,25$(OH)_2$D (section 2.1.3.2), bone ALP (section 2.1.3.3), osteocalcin (section 2.1.3.4), 1-84 PTH (section 2.1.3.5) and iPTH levels (section 2.1.3.6)

3.2.4 Bone Biopsy Micro-Computed Tomography (Micro-CT)

Assessment of bone quantity and microarchitecture by 3D micro-CT was performed as described in section 2.1.5. The variables that define bone quantity included BV/TV, Tb.N, Tb.Sp. The bone quality variables included BS/BV, Tb.Th, Tb.Pf and SMI. Briefly, BS/BV is an indicator of texture and thickness of the trabecular bone, Tb.Pf is an indicator of the surface curvature and SMI is an index of the ratio of trabecular plates to rods.

3.2.5 Bone Biopsy Histology

Preparation of bone biopsies for histomorphometric analyses is described in section 2.1.4. Wall thickness was measured using Haematoxylin and Eosin stain as described in section 2.1.6. This histomorphometric analyses was done only in the fracture cohort.

3.2.6 Bone mRNA Analyses

The method of RNA isolation, extraction and quantification is described in section 2.1.7. Preparation of first strand cDNA was undertaken as described and quantitative real time PCR was performed to determine mRNA expression of CYP27B1, CYP24A1 and VDR.

3.2.7 Radiographic Cortical Bone Analyses

Anterior-posterior plain radiographs of the contralateral side were used to measure average cortical thickness, medullary width and total femoral width as described in section 2.1.8. These radiographs were available only for the fracture cohort.

3.2.8 Statistical Analyses

Statistical analysis was performed using the SPSS statistical Package (IBM SPSS Stats 25, St Leonards, Australia). Some of the variables were not normally distributed,

hence values are expressed as median and interquartile range. Mann-Whitney U test was used to compare the fracture and non-fracture group. Spearman's correlation coefficient was calculated to determine correlations between structural, metabolic and gene expression variables for the entire cohort and separately for the fracture cohort. The metabolic variables which correlated with structural variables were tested through stepwise multiple linear regression analyses to identify metabolic determinants of bone mass and bone quality variables in the fracture cohort. The fracture cohort was divided into 25(OH)D intervals as 10-30 nmol/L, 30-50 nmol/L, 50-70 nmol/L and greater than 70 nmol/I. Independent samples Kruskal–Wallis test was used to compare bone structural and quality variables between the intervals. The significance level was set at 0.05.

3.3 Results

We first present the difference in the fracture and non-fracture group and then discuss the effect of vitamin D on bone parameters in the fracture cohort.

3.3.1 Microarchitecture in Fracture and Non-Fracture Groups

The fracture cohort was significantly older than the non-fracture group, with a median age 87 years vs. non-fracture cohort, where the median age was 62 years. Fracture patients had significantly lower BV/TV and Tb.N compared to non-fractured patients. Fracture patients also demonstrated altered trabecular microarchitecture characterized by significantly lower Tb.Th and higher BS/BV, Tb.Sp, Tb.Pf and SMI indicating the presence of more rod-like trabeculae and fewer plate-like structures consistent with reduced bone strength (table 3.1). The rod-like trabeculae in fracture patients compared to the presence of plate-like trabeculae in the non-fracture patients is illustrated in biopsy images of an age and sex matched non-fracture patient and a fracture patient (figure 3.1 A and B). It is interesting to note that in these illustrative images, despite the fracture patient having higher bone mass as compared to non-fractured patient, the bone quality was significantly inferior in the fracture patient with higher BS/BV and Tb.Pf and lower Tb.Th.

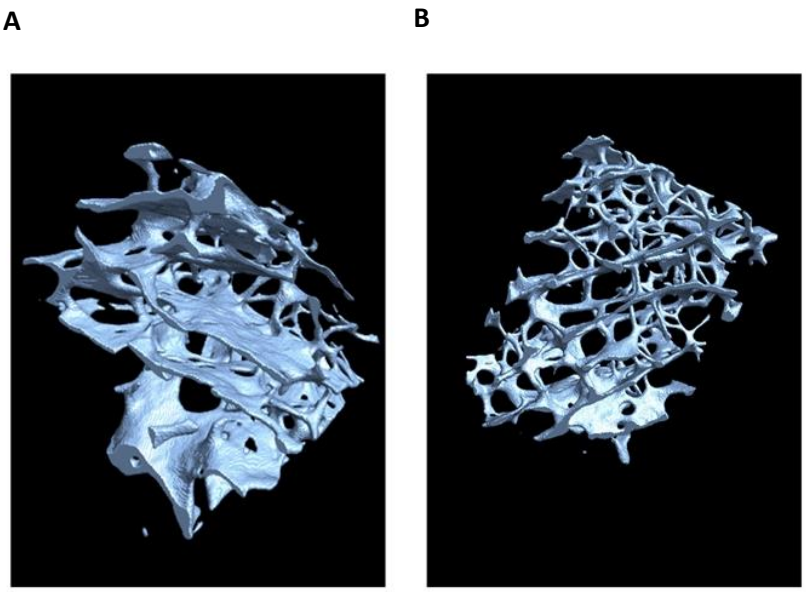

Figure 3. 1 MicroCT images of bone specimens showing trabecular microarchitecture in an age and sex matched (female) (A).non-fractured and (B) fracture patient

A. Non-fractured patient
Age 86-year
BV/TV = 9.96; BS/BV = 22.8mm^{-1}
Tb.Th = 0.16mm; Tb.Pf = 5.2 mm^{-1}

B. Fractured patient
Age 87-year
BV/TV = 10.58; BS/BV = 27.1mm^{-1}
Tb.Th = 0.12mm, Tb.Pf = 6.4 mm^{-1}

Table 3. 1 Trabecular bone microarchitecture of fracture and non-fracture patients (median and range)

Parameter	Fracture patients (n = 103)	Non-fracture patients (n = 27)	Between-group differences P value (Mann-Whitney U test)
Age (years)	87 (63 - 102.7)	61.6 (34.9 - 86.1)	<0.001
BMI	24.4 (14.3-43.9)	32.8 (22.2-47.7)	<0.001
BV/TV (%)	8.9 (2.6-20.4)	15.8 (7.1 - 30.5)	<0.001
BS/BV (mm^{-1})	25.0 (18.4-39.2)	22.4 (16.4-28)	0.001
Tb.Th (mm)	0.14 (0.10-0.19)	0.16 (0.12-0.21)	0.002
Tb.Sp (mm)	0.99 (0.47-2.25)	0.72 (0.43-1.1)	<0.001
Tb.N (mm-1)	0.60 (0.19-1.4)	0.96 (0.52-1.86)	<0.001
Tb.Pf (mm-1)	6.82 (1.57-15.89)	5.29 (-4.02 -8.27)	<0.001
SMI	1.73 (0.91-2.51)	1.65 (0.013-1.98)	0.009

When matched for age, between 60 and 80 years, we noted that the fracture patients had overall lower BV/TV at a given age compared to the non-fracture controls which indicates that fracture patients had lost significant amount of bone mass early in life. Further, age was not correlated with bone mass or bone quality variables in either fracture or non-fracture group and age was not a determinant of any variable such as BV/TV, Tb.Th or BS/BV in the fracture cohort and the non-fractured control group suggesting that the microarchitectural differences observed between the two groups were not due to age differences. The spearman's correlation coefficient between age and bone quantity/quality measures in the fracture cohort were: BV/TV (r = 0.022, p = 0.8), BS/BV (r = -0.005, p = 0.9), Tb.Th (r = 0.075, p = 0.5) and in the non-fractured group were: BV/TV (r = -0.16, p = 0.5), BS/BV (r = 0.05, p = 0.8) and Tb.Th (r = -0.04, p = 0.9).

3.3.2 Metabolic Differences Between Fracture and Non-Fracture Groups

The metabolic profile of fracture and the non-fractured patients is presented in table 3.2. In the fracture cohort, 40% of the patients presented with compromised kidney functions (eGFR<60ml/min per 1.73m^2 body surface area) and 45% of the patients were vitamin D deplete as defined by serum 25(OH)D levels less than 50 nmol/L. PTH levels were significantly higher and osteocalcin levels were lower in the fracture group. Albumin levels, a good marker of nutritional status, were significantly higher in the non-fracture group as

compared to fracture group. Around 63% of the fracture patients had lower albumin (less than 34 g/L), suggesting a poor nutritional status in the fracture group as compared to the non-fracture group .

The levels of 25(OH)D were lower in the fracture cohort, though the differences in the 25(OH)D values between the two cohorts did not reach statistical significance. Serum 25(OH)D correlated positively with circulating $1,25(OH)_2D$ ($r = +0.342$, $p = 0.0004$) in the fracture cohort further confirming substrate insufficiency in this cohort.

Table 3. 2 Metabolic profile of fracture and non-fracture patients (medians and range)

Variable	Fracture patients (n = 103)	Non-fracture patients (n = 27)	Between-group differences p value (Mann-Whitney U test)
eGFR (ml/min/1.73 m^2)	70 (11->90)	86 (29- >90)	<0.001
Ionized calcium (mmol/l)	1.19 (1.03-1.37)	1.21 (1.11-1.31)	NS
Albumin (g/l)	31 (18-44)	41 (23-47)	<0.001
25(OH)D (nmol/l)	52.7 (10-225)	62.8 (20.5-115)	NS
$1,25(OH)_2D$ (pmol/l)	93.8 (18.4-229)	101 (32-284)	NS
PTH (pmol/l)	4.9 (1.1-32.7)	3.9 (1.2-12.8)	0.058
1-84 PTH (pmol/l)	2.8 (0.7-16.2)	1.9 (0.87-5.8)	0.002
Osteocalcin (nmol/l)	2.4 (0.33-21.8)	3.3 (1.7-9.3)	<0.001
Bone ALP (nmol/l)	13.3 (3.5-36.3)	14.5 (5.71-29.9)	NS

3.3.3 Relationship between Vitamin D Levels and Trabecular Bone Quantity, Quality and Cortical Bone Structure in the Fracture Cohort

The fracture cohort was further investigated to understand the influence of varying serum 25(OH)D levels on trabecular bone structure, gene expression and cortical bone expression. Serum 25(OH)D levels did not correlate with the bone structure variables of BV/TV, Tb.Th and Tb.N by either linear analyses ($p > 0.05$, for all) nor when serum 25(OH)D levels were grouped by intervals of 25(OH)D adequacy ranging from deplete to replete (figure 3.2 A–C). However, a trend for serum 25(OH)D levels association with Tb.Th

was observed (r = 0.19, p = 0.06). Neither serum 1,25$(OH)_2$D nor PTH levels correlated with BV/TV, Tb.N or Tb.Th. Wall thickness or MWT, a dynamic marker of completed bone formation and an indicator of the length of time osteoblast work, exhibited a positive linear correlation with 25(OH)D levels (r = 0.810, p < 0.0001) (figure 3.3). MWT trended to be higher in patients with 25(OH)D greater than 70 nmol/L as compared to patients with serum 25(OH)D levels between 30–50 nmol/L (p = 0.021, adjusted p = 0.12) (figure 3.2 D, table 3.3). Although, MWT values also correlated with serum 1,25$(OH)_2$D levels, only serum 25(OH)D determined MWT values in a multiple linear regression model (table 3.4).

BS/BV, a measure of bone texture and a surrogate marker of plate-like trabecular structure, was negatively associated with serum 25(OH)D levels (r = −0.206, p < 0.05), indicative of higher vitamin D levels resulting in more plate-like structures. However, the BS/BV values did not reach statistical significance when divided by serum 25(OH)D intervals (figure 3.2 E). Neither serum 1,25$(OH)_2$D nor PTH correlated with BS/BV (p = 0.9 and 0.8, respectively). With respect to cortical bone measurements, serum 25(OH)D levels correlated negatively with total femoral diameter (r = −0.239, p = 0.018) and trend only for medullary width (r = −0.169, p = 0.09). None of the cortical parameters were associated significantly across vitamin D intervals (figure 3.2 F-H). Neither serum 1,25$(OH)_2$D nor PTH contributed to the variation in any cortical measure.

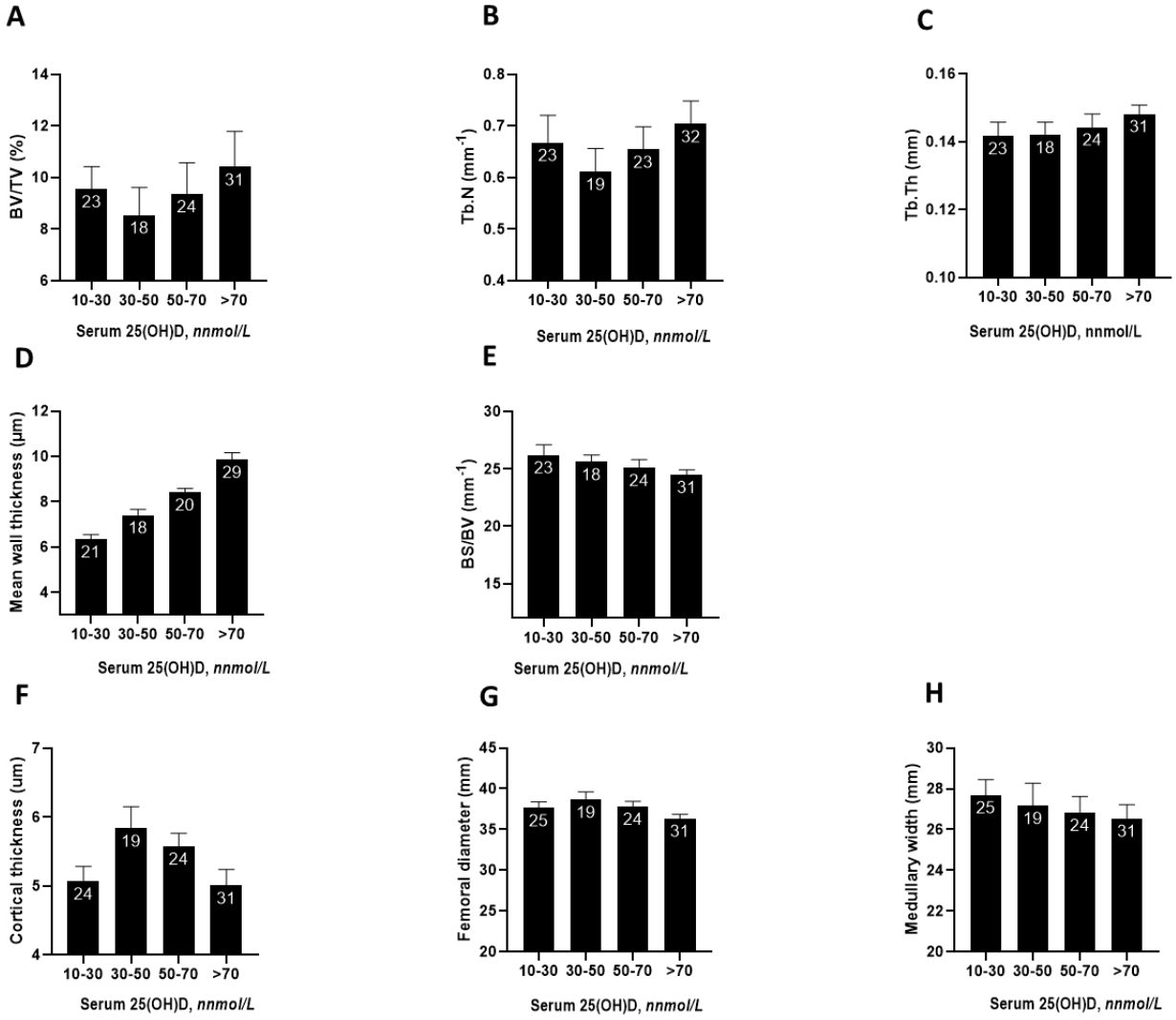

Figure 3. 2 Trabecular and cortical bone structural variables across 25(OH)D intervals with (A) BV/TV (B) Tb.N (C) Tb.Th (D) MWT (E) BS/BV (F) cortical thickness (G) femoral diameter (H) medullary width. Mean +/− SEM; n = 103; * $p < 0.05$ vs. 10–30 nmol/L 25(OH)D interval. The number of patients in each of the 25(OH)D interval is indicated within the respective bar.

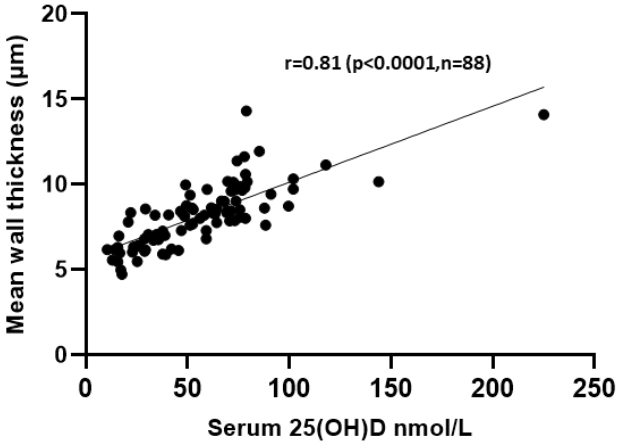

Figure 3. 3 Linear regression between serum 25(OH)D levels and MWT

Table 3. 3 Comparison of MWT across 25(OH)D intervals (Kruskal-Wallis Test)

25(OH)D interval nmol/L (number of patients)	**Median 25(OH)D levels (nmol/L)**	**Test Statistics**	**p value (vs >70 nmol/L)**	**Adjusted p value (vs >70 nmol/L)**
10-30 (21)	6.17	-50.31	<0.001	<0.001
30-50 (18)	7.16	-34.77	<0.001	<0.001
50-70 (20)	8.40	-17.17	0.021	0.12
>70 (29)	9.70			

[a]Significance values have been adjusted by the Bonferroni correction for multiple tests

Table 3. 4 Determinants of MWT using multiple linear regression analyses

Dependent variable	**Independent variables**	**Standardized coefficients (β)**	**p-value**
MWT	25(OH)D	0.745	<0.001
	+ $1,25(OH)_2D$	+0.095	0.19 (NS)
	+5.4		
		Adjusted R^2 = 0.602	p < 0.001

3.3.4 Contribution of CYP27B1 And CYP24A1 mRNA Expression in Bone to Histomorphometric Measures

To determine whether bone-specific expression of vitamin D metabolizing enzymes, CYP27B1 and CYP24A1 contribute to the relationship of serum 25(OH)D with MWT and BS/BV, multiple linear regression analyses were carried out to investigate the variance accounted by these measures. Bone CYP27B1 and CYP24A1 mRNA levels did not contribute to the serum 25(OH)D determination of MWT (table 3.5). However, bone CYP24A1 mRNA contributed positively (p = 0.02) and CYP27B1 mRNA negatively (trend only, p = 0.1) contributed to the serum 25(OH)D determination of BS/BV (table 3.6).

Table 3. 5 Determinants of MWT-multiple linear regression analyses

Dependent variable	Independent variables	Standardized coefficients (β)	p-value
MWT	25(OH)D	0.743	<0.001
	CYP27B1	+0.043	0.66 (NS)
	CYP24A1	+0.064	0.50 (NS)
		+5.1	
		Adjusted R^2 = 0.54	P < 0.001

Table 3. 6 Determinants of BS/BV ratio- multiple linear regression analyses

Dependent variable	Independent variables	Standardized coefficients (β)	p-value
BS/BV	25(OH)D	-0.294	0.012
	CYP27B1	-0.208	0.115
	CYP24A1	+0.306	0.021
		+27.5	
		Adjusted R^2 = 0.104	0.016

3.3.5 Effect of Calcium and Vitamin D Supplements Intake on Bone Measures in The Fracture Cohort

In the fracture cohort, 55% of the patients were not taking any supplement, 3% were taking calcium alone, 9% were taking vitamin D alone and 11% were taking both calcium and vitamin D at the baseline. No records were found for 22% of the patients. Table 3.7 shows differences in serum 25(OH)D levels and structural variables between the various supplement group. Serum 25(OH)D levels were higher in the calcium and vitamin D group as compared to the no supplement group, though the values did not reach statistical significance. Medullary width, an indicator of bone resorption, was significantly lower ($p < 0.0001$) in the patients taking calcium and vitamin D supplement (23.07 ± 1.47mm) at the baseline as compared to patients not taking any supplement (28.34 ± 3.56). Also, Tb.Sp was significantly lower (16%, $p = 0.04$) while Tb.N was higher (21%, trend only, $p = 0.07$) in the calcium and vitamin D group as compared to the no supplement group. None of these variables were different between the patients consuming only vitamin D as compared to control (table 3.7). This is consistent with the clinical observations that both vitamin D and calcium are required to maintain bone health. Further average cortical thickness was 10% higher in the calcium and vitamin D group as compared to no supplement group. Though this number did not reach statistical significance ($p = 0.21$), clinically a 10% higher cortical thickness may be important in preventing from fractures.

Table 3. 7 Baseline supplement intake in the fracture patients (mean ± sd)

Baseline supplement	Number of patients	25(OH)D nmol/L	Tb.Sp mm	Tb.N mm^{-1}	Cortical thickness (mm)	Medullary width (mm)
Calcium and vitamin D [a]	11	63.8 ± 22.7	0.90±0.17	0.75±0.24	5.76±1.01	23.07±1.47
Vitamin D alone [b]	9	69.7 ± 17.2	0.98±0.29	0.73±0.27	5.1±1.13	27.76 ± 3.55
No supplement [c]	57	51.4 ± 26.4	1.07±0.26	0.62±0.21	5.23±1.21	28.34±3.56
T-test summary	a vs c	0.14	0.04	0.07	0.21	<0.0001
	b vs c	0.05	0.48	0.18	0.77	0.65

3.4 Discussion

Clinical studies using sophisticated imaging techniques such as HR-pQCT, finite element modelling or individual trabecula segmentation (ITS) comparing fracture patients and non-fractured controls suggests preferential reduction in trabecular plate bone volume, trabecular plate number, less trabecular connectivity and more rod-like trabecular network in fracture patients as compared to non-fracture controls [207, 446, 447]. The plate to rod ratio is reported to be 20-30% lower in fracture subjects as compared to non-fracture control [207, 447]. Our study adds to the existing evidence and shows significant alterations in trabecular microarchitecture in a large cohort of 103 male and female hip fracture patients as compared to non-fracture patients. Hip fractures patients had significantly lower bone mass parameters such as BV/TV and Tb.N. Further, fracture patients presented with altered trabecular bone micro-architecture with significantly higher BS/BV indicative of more rod-like structure consistent with weaker bone. Tb.Sp and Tb.Pf were higher in the fracture group indicative of disconnected structure characteristic of reduced bone strength. Our study suggests that fragility fracture is associated with preferential loss of thicker plate-like trabecular structures consistent with the literature [207]. Previous studies show that loss of plate-like trabeculae in fracture patients results in reduced bone strength measured as bone stiffness (10%-11% lower), $p < 0.01$ [446]. In our study, the loss of trabecular plate like structures in the fracture group could have resulted in reduced bone strength subsequently leading to fragility fracture. Furthermore, the fracture patients had significantly lower BV/TV at any given age compared to non-fractured controls suggesting that fracture patients had lost significant amount of bone mass at a relatively early age. Fragility fractures in the fracture cohort could be explained by an early loss of bone mass due to the disease (osteoporosis) followed by compromised bone quality/strength (higher BS/BV and lower Tb.Th) later in life. Such a proposal would require further investigations to establish its validity.

Further, in this study we propose an important variable of bone quality–BS/BV. So far, most of the micro-CT studies describe Tb.Th and SMI as a measure of bone quality. The algorithm for measurement of BS/BV and Tb.Th are completely different and hence they

provide information on different aspects of trabecular bone. Surface area and volume are calculated based on 3D subpixel surface rendering by a marching cubes algorithm while Tb.Th is calculated by the sphere-fitting local thickness algorithm [448]. BS/BV informs about bone quality in greater detail and includes assessment of inter-trabecular connectivity. Higher values of BS/BV and Tb.Pf in the fracture group indicate disconnected structure with relatively higher number of rods and these parameters have been reported to be associated with direct measures of bone strength [209]. In our study, we have found preserved connectivity and more plate-like trabecular configuration in the non-fracture control group consistent with higher bone strength. The comparison between fracture and non-fracture group confirm that the mechanism associated with trabecular thinning and conversion of plates to rods in hip fracture patients are distinct from changes occurring in the normal ageing process.

From a preventative point of view, it is important to identify pharmaceutical agents and/or nutrients or dietary patterns that can help retain plate-like structure and hence higher bone strength in human trabecular bone. In the present study, we have demonstrated for the first time in humans that adequate serum 25(OH)D levels along with higher expression of bone mRNA levels of CYP27B1 and lower expression of CYP24A1, consistent with formation of bone-derived $1,25(OH)_2D$ levels, is associated with stronger trabecular bone structure. This is referred to as autocrine activity of vitamin D which is independent of its endocrine actions or circulating $1,25(OH)_2D$. The strong positive relationship between serum 25(OH)D levels and MWT suggests that osteoblastic bone formation was improved in the presence of higher serum 25(OH)D levels. Serum 25(OH)D levels above 50 nmol/L was associated with significantly higher MWT. While it is possible that serum 25(OH)D levels of 70 nmol/L and above, increased MWT further ($p = 0.021$), when we adjusted for multiple comparisons, the significance was lost ($p = 0.2$). This could be due to smaller number of participants per group. BS/BV, an indicator of texture and thickness of bone, was negatively associated with 25(OH)D levels suggesting that patients with adequate vitamin D status had plate-like structure consistent with higher bone strength. Significantly lower BS/BV and Tb.Pf values were reported in a recent clinical

study examining the effect of bisphosphonate treatment on femoral head trabecular bone micro-architecture in non-treated fracture controls as compared to hip-fracture patients. The authors concluded that lower values for both the variables suggest preserved connectivity of trabecular bone in the bisphosphonate group. More importantly, Tb.Pf and BS/BV were strongly correlated with measures of bone strength [209]. Tb.Pf correlated strongly with yield strength ($r = -0.762$, $p = 0.001$) and BS/BV correlated significantly with maximum strength ($r = -0.744$, $p = 0.003$) in both bisphosphonates treated and non-treated fracture group. A lower BS/BV may also signify decrease bone resorption [209]; hence, a lower BS/BV with higher vitamin D levels observed in our study is consistent with structural effects of vitamin D on reducing bone resorption.

We have previously proposed, using animal and cell culture studies, that the direct supply of 25(OH)D to bone is required for local $1,25(OH)_2D$ synthesis [337, 344, 449] and contributes to the regulation of bone formation [332] and resorption [450, 451] processes. Few studies to date have investigated the relationship between 25(OH)D status and local metabolism of vitamin D within human bone. Elderly women with subcapital femur fracture have been shown to exhibit five-times lower levels of bone-derived $1,25(OH)_2D$ levels when compared with aged-matched women without fracture, despite comparable circulating levels of $1,25(OH)_2D$ [452]. We have previously shown evidence for autocrine actions of vitamin D using eleven human biopsies and the relationship of CYP27B1, CYP24A1 and VDR mRNA levels with genes, including vitamin D responsive genes involved in bone remodelling [453]. Furthermore, we have shown that human primary osteoblasts synthesize $1,25(OH)_2D$ from 25(OH)D via CYP27B1 activity, which directly promotes mineralization [335]. While these studies are indicative of the requirement for 25(OH)D to support bone health, the question of changes in human structural bone in relation to serum 25(OH)D were less clear. In the present study, we have demonstrated that adequate serum 25(OH)D levels along with bone mRNA levels, which are higher for CYP27B1 and lower for CYP24A1, is associated with reduced BS/BV, which is indicative of a stronger trabecular bone structure. Our findings concur with a recent clinical study in a Danish cohort of post-menopausal women that showed daily supplementation with vitamin D_3 (2800 IU) resulted in significantly

improved bone strength but did not affect the lumbar spine, hip and whole body aBMD [444]. Finite element analyses was performed to measure bone strength, expressed as failure load (the breaking capacity) and stiffness (resistance to deformation) [444] .

The observation that patients consuming calcium and vitamin D at baseline had significantly lower medullary width and lower Tb.Sp, is consistent with clinical evidence suggesting that both these nutrients work synergistically to improve bone health. Further, a 10% higher cortical thickness in the calcium and vitamin D group may be clinically relevant. However, these findings are limited due to very small number of patients and require further investigation. A 12% lower cortical thickness has been reported in fractured neck of femur patients (n = 143) as compared to age and sex matched controls (n = 298) in a prospective nested case-control study [454].

Given the significant association of serum 25(OH)D levels with bone quality variables, we propose that serum 25(OH)D levels could be explored as one of the future biomarkers of bone quality and potential fracture risk. Based on the results of the present study, we propose 25(OH)D levels of 50-75 nmol/L are required for stronger bones with plate-like trabecular configuration. No impact of vitamin D status on BV/TV suggests that BMD which is often used as an indicator of efficacy of calcium and vitamin D supplementation may not be sensitive to detect changes associated with supplementation of these nutrients. Hence, including bone quality assessments such as using HR-pQCT in future RCT's on calcium and vitamin D supplementation may be more informative.

Chapter 4: Visceral Fat is a Negative Determinant of Bone Health in Obese Postmenopausal Women

"I never did anything by accident, nor did any of my inventions come by accident; they came by work".

Thomas A Edison

4.1 Introduction

The impact of obesity on bone metabolism and risk of fractures has been of major interest, with considerable controversy and debate. Previously, obesity, when measured as BMI, was thought to be protective for bone health as individuals with a higher BMI usually have a higher BMD [351]. However, the risk of fractures is not low in this population with several studies demonstrating that obesity, when measured as total and regional adipose mass, is associated with a higher risk of some site-specific fractures [112, 139] as discussed previously in section 1.10.3. Furthermore, meta-analyses of prospective studies suggest that a 0.1 unit increase in waist: hip ratio increases the risk of hip fracture by 3% [392] indicating that higher central or abdominal fat is associated with a higher risk of hip fracture. Consistent with this, studies using high resolution CT imaging demonstrate that VAT is more strongly associated with poor bone microarchitecture and reduced bone strength than other fat regions [354, 355, 357, 396]. Bredella et al. have reported that VAT was a negative predictor of microarchitecture and mechanical properties in young obese men [355] and a negative predictor of lumbar spine BMD in premenopausal women [354]. In addition, healthy premenopausal women with the highest tertile of trunk fat levels, which approximates to VAT, exhibited inferior bone quality and stiffness as well as reduced bone formation in transiliac bone biopsies despite reporting normal BMD [357]. These studies, which suggest that higher amount of VAT is detrimental to bone quality and strength are consistent with reports of the harmful effect of higher VAT even when BMI is within the normal range [396]. All these studies have been discussed in detail in chapter 1 (section 1.10.4). Even when a longitudinal study associate higher VAT with higher BMD and improved bone microarchitecture, when these associations were adjusted for BMI, VAT was no longer a positive determinant of bone parameters [399] suggesting that the relationship was driven primarily by overall obesity rather than due to VAT. This is consistent with findings reported in a recent Mendelian randomization study in 594 post-menopausal Korean women which showed that abdominal adiposity (defined as BMI adjusted WC, a surrogate for VAT) was neutral to BMD in post-menopausal women [455].

The possible mechanism by which VAT mediates detrimental effect on bone are

not fully elucidated. Some investigators have shown that higher VAT is associated with lower bone formation markers and reduced IGF-1 levels in premenopausal women [354, 357] and with decreased GH and testosterone levels in men [355].

In this chapter, we present the baseline findings of the relationships between varying BMI and VAT levels with BTMs, total body BMD, vitamin D metabolites and PTH levels in post-menopausal women.

4.2 Study Participants and Methods

4.2.1 Participant Recruitment

The methodology is given in detail in chapter 2 (section 2.2). Briefly, healthy post-menopausal women with BMI < 25 kg/m^2 and BMI > 27 kg/m^2 were recruited through advertisements placed in the local newspapers, social media and radio between July-November 2017. The criterion for BMI was chosen to enable selection of women with varying VAT amounts. All interested women were asked to contact the clinical trial facility at the University of South Australia, by telephone or email. Upon receipt of email or phone call, interested volunteers were sent the participant information sheet which had complete details of the study, inclusion and exclusion criteria, and risk involved in participating. The inclusion criteria were post-menopausal (at least 5 years post menopause), not taking calcium or vitamin D supplements, or, if so, agreeing to stop these for 4 weeks before entering the trial. Women taking any medications known to affect bone metabolism, diabetes and a fracture in the past 12 months were excluded from the study. Volunteers who expressed interest in participation were invited to the clinical trial facility for a screening visit to assess their suitability for the study. The participants who met the BMI and other inclusion criteria were provided details about the study and informed consent was obtained. Participants were requested to complete an information sheet that provided details on current medication and illness history. Seventy-seven healthy normal (BMI < 25 kg/m^2, n = 27) and overweight (BMI > 27 kg/m^2, n = 50) post-menopausal women with no osteoporosis, no history of low trauma fractures and no causes of potential bone loss were

selected for the study. Approval was obtained from University of South Australia human ethics committee and ratified by the independent Royal Adelaide Hospital human ethics committee before starting the trial. The trial was registered at the Australia New Zealand Clinical Trial Registry (ACTRN12617000779370).

4.2.2 BMD and Body Composition Analyses

All participants underwent body weight measurements to the nearest 0.1 kg using standardized scales and standing height to the nearest 0.1 cm using a stadiometer. Hip and WC were recorded to the nearest 0.1 cm using a non-stretchable measuring tape. WC was measured at midpoint between the top of the iliac crest and the lower margin of the last rib . Hip circumference was measured at the largest circumference of the buttocks . Total body DXA scans were acquired using the Lunar iDXA (GE Healthcare, Madison, WI) to measure whole-body fat mass, lean mass, android fat, gynoid fat, A:G ratio and total body BMD. CoreScan software was used to measure SAT and VAT over the android region as described previously in section 2.2.2.1.

4.2.3 Biochemical analyses

Serum samples were analyzed by chemiluminescent immunoassay (Diasorin LiaisonXL) for 25(OH)D levels, $1,25(OH)_2D$ levels, 1-84 PTH levels, osteocalcin levels and bone ALP levels as per methods explained in chapter 2 (section 2.1.3). Serum calcium, phosphorus and ALP levels were measured by Thermo Fisher Scientific automated Kone lab analyzer as describe previously in section 2.2.4.3. Plasma samples were analyzed for serum CTX-1 levels using fully automatic IDS-iSYS immunodiagnostic system using 2 step chemiluminescence immunoassay as described in section 2.2.4.1. CTX-1 and 1-84 PTH levels were measured in fasting blood specimens taken on three different days and average value was considered for analyses. One participant had CTX-1 levels below the reportable range of the assay (< 33 ng/L) and was excluded from data-analyses.

4.2.4 Statistical Analyses

Normality was assessed with the use of histograms and Shapiro-Wilk test. Most of the variables (BMD, BMI, WC, CTX-1 levels, bone ALP levels, $1,25(OH)_2D$ levels, 1-84 PTH levels) were not normally distributed, hence Spearman's coefficient of correlation was used to describe the relationships between body fat variables and bone markers. To investigate the effect of VAT on bone health, participants were divided into VAT quartiles and non-parametric Kruskal-Wallis test was used to compare the quartiles. Multiple linear regression models were used to investigate determinants of bone and biochemical variables. All the analyses were carried out using SPSS (IBM SPSS Stats 25) and $p < 0.05$ was considered significant.

4.3 RESULTS

4.3.1 Participant Characteristics

The median age of the participants was 65.5 years (IQ range 61.9-68.5 years). 36% women had normal BMI (<24.9 kg/m^2, n = 27), 29% women were overweight (BMI 27-29.9 kg/m^2, n = 22) and 36% were obese (>30 kg/m^2, n = 27). Median android fat percentage was 49% (range: 19.2-67.8%) and gynoid fat was 47% (range: 25.5-58.5%). Median VAT volume was 946 cm^3, SAT volume was 1780 cm^3 and CTX-1 levels were 417 ng/L. The amount of VAT varied significantly even within a narrow BMI range. VAT volumes in the lean group (BMI < 25 kg/m^2) ranged from 91-1050 cm^3 and in the overweight group (BMI >27 kg/m^2) ranged from 425-3392 cm^3. Hence, to investigate the effect of VAT, participants were divided into VAT quartiles and their characteristics and biochemical profile as per VAT quartiles are described in table 4.1 and table 4.2, respectively. There was no difference in participant's age across quartiles, BMI was normal in the first quartile (<25 kg/m^2) and all measures of adiposity such as BMI, WC, android fat and SAT as well as BMD increased with increasing VAT volume. VAT and WC correlated positively and significantly ($r = 0.855$, $p < 0.001$). The median WC was less than <88 cm, recommended cut-off reference [436] in the first and second quartile and increased significantly across the third and fourth quartile.

Table 4. 1 Participant characteristics by VAT Quartiles; median (inter-quartile range), n = 19/quartile

VAT, cm^3	**Quartile 1 231 (190-425)**	**Quartile 2 780 (690-874)**	**Quartile 3 1208 (1050-1302)**	**Quartile 4 1723 (1615-2226)**	**p-value <0.0001**
Age, y	65.7 (61.8-68.8)	65.4 (61.4-68.1)	66.3 (64.2-69)	64.8 (61.2-67.3)	0.4 (NS)
Weight, cm	57.7 (48.2-63.8)	68.5 (62.7-78)	74.8 (72.1-81)	93 (83.3-100.2)	<0.0001
Height, cm	159.4 (155.1-162.6)	165.6 (156.8-169.5)	163.3 (157.4-165.8)	161.6 (158.2-164.5)	0.15 (NS)
BMI, kg/m^2	22.9 (20.2-24.0)	27.3 (24.0-27.9)	28.0 (27.2-29.5)	35.5 (32.0-39.5)	<0.0001
WC, cm	75 (71-77.5)	85.6 (81.5-94.7)	93.5 (88-96)	109.5 (102-118)	<0.0001
Total fat mass, kg	18.9 (13.0-22.3)	28.1 (24.1-31.2)	32.6 (29.1-36.5)	44.9 (37.9-49.8)	<0.0001
Total lean mass, kg	35.1 (32.9-38.6)	39.7 (34.5-43.4)	38.6 (36.3-41.2)	45.1 (39.7-48.6)	<0.0001
Android fat mass, kg	4.1 (3.4-4.4)	5 (4.7-5.9)	5.8 (5.3-6.1)	7.6 (6.8-8.8)	<0.0001
Android lean mass, kg	2.7 (2.5-2.9)	2.9 (2.6-3.2)	2.7 (2.5-3.2)	3.3 (2.9-3.9)	<0.0001
Android fat mass, kg	1.4 (0.8-1.5)	2.3 (1.9-2.6)	2.9 (2.6-3.2)	4.2 (3.8-4.8)	<0.0001
Android fat (%)	33.7 (24.8-36.9)	44.8 (42.2-49.9)	52.1 (46.4-54.2)	56.5 (54.7-58.8)	<0.0001
Gynoid fat (%)	39.5 (33.8-43.8)	47.4 (42.6-48.8)	47.8 (44.1-49.9)	50.1 (48.2-53.1)	<0.0001
SAT, cm^3	1178 (759-1407)	1714 (1321-2107)	1817 (1605-2178)	2510 (2138-3376)	<0.0001
A/G ratio	0.88 (0.74-0.94)	1.0 (0.9-1.04)	1.1 (1.0-1.17)	1.13 (1.08-1.17)	<0.0001
BMD, g/cm^2	1.04 (0.91-1.14)	1.11 (0.99-1.14)	1.12 (0.99-1.2)	1.17 (1.10-1.23)	0.016

Table 4. 2 Participant biochemical profile by VAT quartiles; median (inter-quartile range)

n = 19/quartile

VAT, cm^3	**Quartile 1 231(190-425)**	**Quartile 2 780(690-874)**	**Quartile 3 1208 (1050-1302)**	**Quartile 4 1723 (1615-2226)**	**p-value <0.001**
Calcium mmol/L	2.34 (2.28-2.41)	2.35 (2.32-2.38)	2.36 (2.26-2.41)	2.33 (2.29-2.37)	0.9
Phosphorus mmol/L	1.21 (1.09-1.23)	1.18 (1.11-1.27)	1.16 (1.14-1.23)	1.08 (0.96-1.21)	0.2
ALP U/L	56.6 (48.7-68.2)	69.4 (57.6-74.8)	60.9 (54.6-89.4)	77.7 (60.8-91.6)	0.007
25(OH)D nmol/L	72.2 (61.5-91.6)	67.7 (47.8-77.1)	71.5 (51-80.8)	57.4 (41.1-73.2)	0.029
$1,25(OH)_2D$ pmol/L	126 (107-157)	126 (102-141)	116 (98.5-154)	117 (106-138)	0.767
1-84 PTH pmol/L	2.4 (2.2-2.6)	2.5 (2.1-3.5)	2.8 (2.2-3.5)	3.4 (3.0-4.1)	0.002
Osteocalcin nmol/L	3.8 (3.4-4.4)	4.1 (3.5-4.5)	3.6 (2.9-4.4)	3.3 (2.9-3.5)	0.025
Bone ALP µg/L	11.6 (9.4-13.1)	13.1 (11.1-15.8)	12.4 (9.2-17.3)	14.1 (9.6-19.6)	0.378
CTX-1 ng/L	454.2 (397.5-640.6)	476.5 (346.2-701.2)	402.4 (320.5-615.7)	314 (248-418)	0.041

Osteocalcin levels and bone ALP levels correlated positively with CTX-1 levels ($r = 0.679$, $p < 0.001$ and $r = 0.401$, $p < 0.001$, respectively). Osteocalcin and bone ALP levels also correlated positively with each other ($r = 0.235$, $p = 0.041$). The correlations between body fat measures and bone markers is presented in table 4.3. CTX-1 and osteocalcin levels corelated negatively with BMI, WC, VAT and SAT but no correlation with percent gynoid fat was observed. Bone ALP levels showed no association with body fat variables (table 4.3). No association was observed between whole body lean mass and bone markers.

Table 4. 3 Spearman's correlation between body fat measures and metabolic markers

Variables		25(OH)D nmol/l	1-84 PTH pmol/l	Osteocalcin nmol/l	CTX-1 ng/l	Bone ALP µg/l	ALP U/L	Calcium mmol/l	Phosphorus mmol/l
BMI	r	-0.30	0.39	-0.31	-0.32	0.16	0.32	0.018	-0.28
	p	0.008	0.001	0.007	0.005	NS	0.004	NS	0.014
WC (cm)	r	-0.22	0.33	-0.30	-0.29	0.086	0.24	0.07	-0.28
	p	NS	0.003	0.008	0.010	NS	0.038	NS	0.016
VAT (cm^3)	r	-0.24	0.37	-0.35	-0.36	0.18	0.37	0.002	-0.24
	p	0.034	0.001	0.002	0.002	NS	0.001	NS	.041
SAT (cm^3)	r	-0.34	0.36	-0.23	-0.26	0.20	0.32	-0.05	-0.17
	p	0.003	0.002	0.042	.025	NS	0.004	NS	NS
Gynoid fat (%)	r	-0.24	0.3	-0.16	-0.20	0.13	0.22	-0.05	-0.28
	p	0.035	0.008	NS	0.076	NS	0.06	NS	0.014

4.3.2 Effect of VAT on Bone Remodelling Markers

CTX-1 levels in this cohort ranged from 138-968 ng/L and women in the highest VAT quartile had significantly lower CTX-1 levels ($p < 0.05$) as shown in table 4.2. Predictors of CTX-1 levels as determined by linear regression analyses were VAT which accounted for 8% variability ($p = 0.008$), WC accounted for 4.4% of the variability ($p = 0.037$) and BMI accounted for 5.5% of the variability ($p = 0.023$). SAT levels, though correlated with CTX-1 levels, were not a significant predictor of CTX-1 levels. To investigate the independent predictors of CTX-1 levels by fat distribution, stepwise multiple linear regression analyses was performed with BMI, VAT, SAT and WC as independent variables. VAT levels were the only significant body fat predictor of CTX-1 levels ($\beta = -0.302$, $p = 0.008$) while BMI, SAT and WC were removed from the model. Further, circulating $1,25(OH)_2D$ levels were a significant positive predictor of CTX-1 levels ($\beta = 0.404$, $p < 0.001$) and along with VAT levels and accounted for 23% of the variability in CTX-1 levels (table 4.4). Furthermore, VAT levels ($\beta = -0.277$, $p < 0.05$) and $1,25(OH)_2D$ levels ($\beta = 0.311$, $p < 0.05$) were independent predictors of osteocalcin levels and together accounted for 17% of the variability as determined by stepwise modeling (table 4.5).

Table 4. 4 Determinants of CTX-1 levels- stepwise multivariate regression

Independent predictors	Standardized coefficient (β)	t	p
$1,25(OH)_2D$	0.404	3.980	<0.001
VAT	-0.263	-2.591	0.012
Constant	193	1.932	0.057

Independent variables added: BMI, VAT, SAT, WC and $1,25(OH)_2D$

Table 4. 5 Determinants of osteocalcin levels - stepwise multivariate regression

Independent predictors	Standardized coefficient (β)	t	p
1,25(OH)$_2$D	0.311	2.940	0.004
VAT	-0.277	-2.617	0.011
Constant	2.89	6.341	<0.001

Independent variables added: BMI, VAT, SAT, WC and 1,25(OH)$_2$D

4.3.3 Effect of VAT on Total Body BMD

BMD increased across VAT quartiles (table 4.1). BMD correlated positively with all fat measures and negatively with bone markers. Spearman correlation coefficients between BMD and body composition variables were: BMI ($r = 0.600$, $p < 0.001$), whole body lean mass ($r = 0.502$, $p < 0.001$), whole body fat mass ($r = 0.568$, $p < 0.001$), WC ($r = 0.421$, $p < 0.001$), VAT volume ($r = 0.432$, $p < 0.001$), abdominal SAT volume ($r = 0.551$, $p < 0.001$) and percent gynoid fat mass ($r = 0.414$, $p < 0.001$). CTX-1 levels, osteocalcin levels and 1,25(OH)$_2$D levels were negatively correlated with BMD while no association between bone ALP levels and BMD was found. VAT accounted for 18% of the variability in BMD in univariate analyses. To investigate the independent predictors of BMD, stepwise multiple linear regression analyses was carried out with BMI, lean mass, SAT, WC, VAT and CTX-1 levels, percent gynoid fat and 1,25(OH)$_2$D levels entered the model. BMI, CTX-1 and VAT levels were the only independent predictors of BMD and together accounted for 48% of the variability in BMD ($p < 0.001$), and the remaining variables did not significantly contribute to the model. While VAT correlated positively with BMD in linear regression analyses, it was a negative predictor of BMD when controlled for BMI ($\beta = -0.368$, $p = 0.017$) (table 4.6). Influence of multi co-linearity between BMI and fat variables was assessed using the variance inflation factor (VIF) and the value was less than 3.5.

Table 4. 6 Determinants of BMD- stepwise multiple regression analyses

Independent predictors	Standardized coefficient (β)	t	p
BMI	0.871	5.833	<0.001
CTX-1	-0.279	-3.198	0.002
VAT	-0.368	-2.435	0.017
CONSTANT	0.764	11.383	<0.001

Independent variables added: BMI, lean mass, SAT, WC, VAT and CTX-1, percent gynoid fat and 1,25(OH)$_2$D

4.3.4 Effect of VAT on Vitamin D Metabolites

The median 25(OH)D levels were 68.6 nmol/L (range 24-135 nmol/L) and the levels decreased across VAT quartiles ($p < 0.05$, table 4.2). Women in the highest VAT quartile (Q4) had the lowest 25(OH)D levels. Serum 25(OH)D levels were significantly and negatively associated with measures of total adiposity and central fat (except WC, $p = 0.052$, table 4.3). Median 1,25(OH)$_2$D levels in this cohort was 119 pmol/L (range 76-220 pmol/L) and were similar across VAT quartiles. Interestingly, while 25(OH)D levels correlated negatively with PTH levels ($r = -0.43$, $p < 0.001$; figure 4.1) and accounted for 20% of the variability in PTH levels, 25(OH)D levels did not show any correlation with BMD or bone markers. Circulating 1,25(OH)$_2$D levels correlated negatively with BMD ($r = -0.299$, $p = 0.009$) and positively with CTX-1 levels ($r = 0.370$, $p = 0.001$) and osteocalcin ($r = 0.305$, $p = 0.007$).

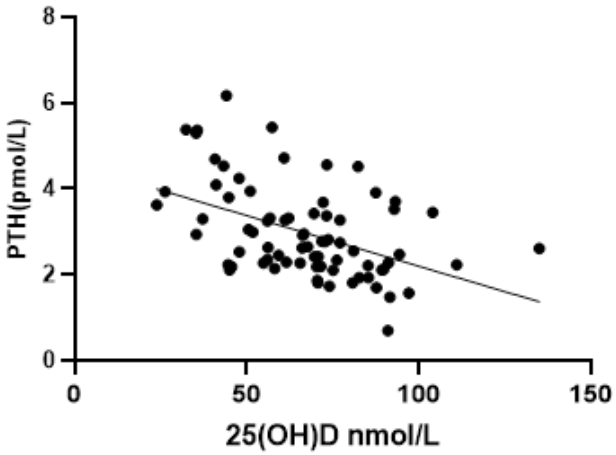

Figure 4. 1 Linear regression analyses between 25(OH)D and PTH levels

4.3.5 Effect of VAT on PTH Levels

The median 1-84 PTH levels in this cohort were 2.63 pmol/L (range 0.57-6.02 pmol/L). 13% of the women had elevated PTH levels (>3.9 pmol/L). The PTH concentration was significantly different between VAT quartiles ($p = 0.002$) and women with the highest VAT (Q4) had highest PTH levels. Serum PTH levels were positively associated with all measures of body fat (table 4.3) but did not correlate with total body BMD or bone markers. VAT accounted for 13% variability in 1-84 PTH levels in univariate analyses. Stepwise multiple regression modeling was conducted to investigate independent predictors of 1-84 PTH levels with BMI, VAT, SAT, gynoid fat, WC, total calcium and 25(OH)D entered the model. 25(OH)D and VAT levels together accounted for 24% variability in PTH ($p < 0.001$) and rest of the variables were removed from the model (table 4.7).

Table 4. 7 Determinants of PTH levels - stepwise multivariate regression

Independent predictors	Standardized coefficient (β)	t	p
25(OH)D	-0.361	-3.455	0.001
VAT	0.274	2.620	0.011
CONSTANT	3.727	8.501	<0.001

Independent variables added: BMI, VAT, SAT, percent gynoid fat, WC, total calcium and 25(OH)D

4.3.6 Effect of VAT on Calcium, ALP and Phosphorus

All participants had normal serum calcium levels (2.35 mmol/L, IQ range 2.29-2.39 mmol/L), phosphorus levels (1.17 mmol/L, IQ range 1.09-1.22 mmol/L) and ALP levels (63.97 U/L, IQ range 55.59-77.71 U/L). There was no correlation between body fat measures and calcium and the levels were similar across VAT quartiles. Phosphorus levels were negatively associated with BMI, VAT and WC (table 4.3) but the levels were not significantly different across VAT quartiles (table 4.2). ALP levels were positively associated with VAT levels (table 4.3).

4.4 DISCUSSION

Although, previous studies have shown that bone turnover is suppressed in obese premenopausal women and obese men [354, 355, 357], this is the first study to characterize relationships between VAT and bone markers, total body BMD, 25(OH)D levels and PTH levels in post-menopausal women with varying BMI levels. Higher VAT levels were associated with reduced bone resorption (CTX-1 levels) and turnover (osteocalcin levels) markers, lower serum 25(OH)D levels and higher PTH levels. Bone ALP levels, a bone formation marker, were not associated with VAT levels.

Positive correlations between VAT and BMD in this cohort are largely explained by higher BMI as after controlling for the effect of BMI, VAT become a negative predictor of BMD. This is consistent with other studies that have shown VAT are most strongly

associated with lower BMD and/or compromised bone structure [354, 396, 456] particularly after controlling for BMI or weight [354, 456, 457]. This was true for all other measures of central adiposity suggesting that BMI assessment may not necessarily account for body fat distribution and central adiposity, which can differ across populations and can vary significantly within a narrow range of BMI [436] . NHANES 1994-2004 data reported that around 24% of US adults with normal BMI presented with metabolic abnormalities suggesting that BMI does not capture the risk associated with higher adiposity and distribution of fat mass [458]. WC is the most widely used indicator of VAT in clinical settings [459]. Hence, we conducted multiple linear regression analyses to determine if WC is equally predictive of CTX-1 levels, osteocalcin levels and total body BMD. However, the results suggest that VAT is the strongest independent determinant of these bone variables as WC was removed from regression analyses. This raises the notion that in clinical settings, VAT may be a more sensitive indicator of bone health in addition to WC in obese postmenopausal women who might otherwise present with higher BMD. Future studies investigating the impact of VAT on bone microarchitecture and strength in post-menopausal women will shed further light on this hypothesis.

Our findings of reduced CTX-1 and osteocalcin levels are consistent with reports of reduced bone resorption and turnover in pre-menopausal women [354, 357] with higher VAT and suggest that obese women with higher VAT remain in a suppressed state of bone turnover even after menopause. A lower bone resorption possibly is the cause of higher BMD observed in women with higher VAT. Furthermore, our observation is consistent with reports of reduced CTX-1 and osteocalcin levels in overweight men and women while bone formation markers PINP and bone ALP levels were similar across BMI groups [422]. Although in other studies higher urinary CTX-1 levels has been observed in obese Egyptian postmenopausal women [460], differences in CTX-1 assays [461, 462] and the absence of a urinary creatinine adjustment for urinary CTX-1 levels [86] as per guidelines for standardized bone assays [87] make biological comparisons difficult to assess. Lower osteocalcin levels observed in this study may be indicative of tight coupling between bone resorption and formation as bone resorption is also reduced in women with higher VAT.

The findings of lower osteocalcin levels in obese post-menopausal women is consistent with numerous previous studies [422, 460, 463, 464] and our data indicates that VAT is an independent predictor of osteocalcin levels. Reduced osteocalcin levels in obese women has been previously associated with increased risk for diabetes and elevated fasting plasma glucose values . Serum osteocalcin levels were also found to be negatively correlated with waist: hip ratio, VAT, serum fasting insulin and homeostasis model assessment of insulin resistance (HOMA-IR) in post-menopausal women [464]. Further, there is evidence to suggest that obese volunteers with suppressed CTX-1 and osteocalcin levels have fasting insulin levels twice that of lean sex and age matched controls [465]. Also, it has been reported that higher VAT resulting in insulin resistance results in lower CTX-1 and osteocalcin levels independent of BMI or total adiposity [466]. Insulin resistance has been associated with reduced bone strength in otherwise healthy obese (non-diabetic) post-menopausal women [467]. Hence, it is plausible that women with higher VAT may have insulin resistance which could result in altered bone microarchitecture and reduced bone strength. This could be a plausible mechanism of higher fracture risk at some sites in women with higher central adiposity despite higher BMD. We hypothesize that lower bone turnover and resorption in women with higher VAT may result in accumulation of unrepaired microdamage resulting from repeated loads applied daily to the bone which further results in lower quality bone. However, this requires further investigation.

Positive correlation between ALP and fat measures such as BMI, WC, VAT is consistent with the literature and may be indicative of ALP release from the adipose tissue [468] or could be reflective of the effect of liver fat causing mild cholestasis.

Lower 25(OH)D levels observed in obese women are consistent with many other studies [413, 417, 423, 424]. Several reasons for lower 25(OH)D levels in obese individuals have been postulated, including sequestration of vitamin D by adipose tissue making it less bioavailable [417]. Other factors such as lower cutaneous synthesis with age [425] may also contribute to the lower 25(OH)D levels. However, most of the conclusive evidence suggests that greater volume of distribution in obese individuals results in lower 25(OH)D levels [418, 422]. Accordingly, the effect of dietary vitamin D supplementation have been found

to be BMI dependent [426, 427] and hence dietary vitamin D therapy needs to be adjusted for body size to achieve the desired serum 25(OH)D concentrations and to suppress PTH levels. The Endocrine Society Clinical Practice Guideline also suggest 2–3 times higher vitamin D dose is required in obese subjects [428]. In the present study, despite lower 25(OH)D levels in women with higher VAT, no associations between vitamin D and bone measures (BMD or bone markers) was observed. This is consistent with what few other have reported [330, 422, 469]. No relationship between 25(OH)D levels and bone variables could be due to sufficient vitamin D levels in the majority of the study participants, with only 24% of the participants having 25(OH)D < 50 nmol/L, the cut-off level considered to be vitamin D insufficient [470] and the median 25(OH)D levels even in the highest VAT quartile was 57 nmol/L. While we have not observed a deleterious effect of lower 25(OH)D levels on total body BMD or bone markers in this cohort, in clinical settings, vitamin D supplementation in order to lower PTH levels is considered beneficial for bone health [19, 30, 471]. Our finding of inverse association between 25(OH)D and PTH levels as reported by many others [31, 310, 422, 472, 473] and that VAT and 25(OH)D levels account for 24% variability in 1-84 PTH levels raises the notion that in clinical settings maintaining adequate serum 25(OH)D levels (either through sunlight exposure or dietary intake or from supplements) may be required to suppress PTH levels and perhaps improved bone quality in obese post-menopausal women. Chapuy et al., [472] have reported a 78 nmol/L cut-off of serum 25(OH)D levels to maintain PTH levels, Rejnmark and colleagues [474] report a 25(OH)D cut-off of 82 nmol/L below which PTH levels start to rise. Some investigators advocate no vitamin D related set-point threshold for PTH and suggest that higher levels of 25(OH)D should be explored for improved bone health [31]. We have shown that higher serum vitamin D levels (greater than 50 nmol/L) are associated with improved bone quality measures without changes in bone quantity variables [475]. Inferior bone microarchitecture and reduced bone strength have been reported in young men and premenopausal women with higher VAT despite normal BMD [354, 355, 357]. Putting these together, we hypothesize that another plausible reason for poor bone microarchitecture in women with higher VAT could be mediated through lower 25(OH)D levels, however we have not tested this yet.

A negative association between $1,25(OH)_2D$ levels and BMD and positive correlation with CTX-1 and osteocalcin levels suggests that higher circulating $1,25(OH)_2D$ levels may be determinantal to bone health which is consistent with the literature [330] and the notion that $1,25(OH)_2D$ may also directly increase bone resorption through its well-recognized role on RANKL-RANK-OPG signalling pathway in concert with PTH [255, 476]. We report that obesity per se does not seem to effect $1,25(OH)_2D$ levels as the levels were not significantly different across VAT quartiles. Others have reported lower $1,25(OH)_2D$ levels in obese as compared to non-obese males and females [411, 477]. It is important to note that in the studies that reported lower $1,25(OH)_2D$ levels in young obese males and females have shown no difference in $1,25(OH)_2D$ levels in individuals with BMI< 25 kg/m^2 and BMI 25-29.9 kg/m^2 [477]. Given that the median BMI in this cohort was 28 kg/m^2, our results seem to be consistent with the above studies and suggest that $1,25(OH)_2D$ levels are not usually elevated in obese male and females [411]. Some of the older studies have reported higher levels of $1,25(OH)_2D$ in young males and females [412, 414]. However, these studies employed radioreceptor assay for measurement of $1,25(OH)_2D$ levels which have several limitations and have been reported to give widely divergent results as compared to the more specific radioimmune assay [478] used in the recent studies [16, 17] as well in the present study. It is important to note that while $1,25(OH)_2D$ levels did not vary across the VAT quartiles, they were an independent positive predictor of CTX-1 and osteocalcin levels. Hence, in post-menopausal women with higher VAT levels, $1,25(OH)_2D$ levels may be an equally important and independent determinant of bone health.

Elevated PTH levels in the highest VAT quartile levels could be related to lower serum ionized calcium levels as reported previously [479]. We measured total calcium levels in this study and there was no significant difference in the values across VAT quartiles. No difference in ionized and total calcium levels have been reported in obese and non-obese white men and women while PTH levels were higher in the obese group [412]. Further, in healthy young men and women (n=1050), it has been reported that PTH levels correlated more tightly with 25(OH)D ($r = -0.249$, $p = 0.0001$) concentrations than ionized calcium levels ($r = -0.105$, $p = 0.0001$) which highlights the influence of 25(OH)D levels on

PTH secretion [480]. In the present study, in addition to 25(OH)D levels, VAT was an independent contributor of PTH levels. A possible explanation on the links between VAT-PTH may arise from the fact that VAT secrete higher level of proinflammatory cytokines such as IL-6, IL-8, CRP [350] whether any of these factors influence PTH secretion in obesity requires further investigation.

In conclusion, higher VAT is associated with reduced bone turnover and resorption in postmenopausal women. Further, postmenopausal with higher central fat had lower 25(OH)D and elevated 1-84 PTH levels. These findings have important clinical implications. The latest data on the prevalence of obesity among Australians suggests that about two-thirds (67.0%) of Australian adults are overweight or obese (12.5 million people) [433] . Given the rising prevalence of obesity in Australia and higher fracture risk at certain sites in obese individuals [112, 348, 363, 364, 380, 386, 481-484], the association between amount of VAT, PTH levels and 25(OH)D levels on bone structure and quality in obese post-menopausal women warrants further investigation. Because bone turnover and bone resorption are already lower in obese as compared to non-obese post-menopausal women, another important question to investigate would be whether reduction in CTX-1 levels through anti-resorptive treatment (anti-resorptives are used to treat post-menopausal osteoporosis and act by suppressing CTX-1 levels) will be effective for fracture prevention in obese postmenopausal women. This question could be partly addressed by reviewing the data on the efficacy of bisphosphonate (a general class name for all antiresorptive drugs) therapy for fracture prevention in postmenopausal women with type 2 diabetics, which is also a state of reduced bone resorption, turnover and higher BMD [79, 485] as observed in the current study in post-menopausal women with higher VAT levels. A post hoc subgroup analysis of combined individual-level data from two randomized placebo-controlled trials, the Fracture Intervention Trial (FIT) of alendronate (ALN) and the Health Outcomes and Reduced Incidence with Zoledronic Acid (ZOL) Once Yearly-Pivotal Fracture Trial (HORIZON-PFT) showed that bisphosphonates reduce fracture risk in postmenopausal women with type 2 diabetes [486]. Further, in the present study, we report a negative correlation between BMD and CTX-1 levels, suggesting that suppressing CTX-1 levels would

be beneficial for bone health.

So far, there is no data to show whether calcium supplementation, which is the first line of therapy for prevention of postmenopausal osteoporosis, either through diet or supplements, would be effective in reducing CTX-1 levels in postmenopausal women with higher VAT who have lower CTX-1 levels as compared to the postmenopausal women with lower VAT. The next chapter is an attempt to provide some clarity on this aspect.

Chapter 5: Visceral Fat Levels do not Affect Acute CTX-1 Suppression with Calcium Supplementation in Post-Menopausal Women - a Randomised Cross-Over Study

"Somewhere, something incredible is waiting to be known."

Carl Sagan

5.1 Introduction

Dietary calcium requirements are increased in postmenopausal women largely due to age-related decline in calcium absorption, higher calcium excretion and loss of protective action of estrogen on bone [117, 133]. A higher intake of calcium in post-menopausal women may offset some of these biochemical and metabolic changes [15, 16, 269]. Several studies have consistently shown that an acute oral dose of calcium ranging between 500-1000mg is effective in reducing CTX-1 levels with a concomitant reduction in PTH levels in both premenopausal [50] and post-menopausal women [51, 52, 66, 116, 118, 119, 264, 266, 271].

We have shown in chapter 4 that postmenopausal women with higher VAT have reduced CTX-1 and elevated PTH levels as compared to women with lower VAT. An intriguing question arising from these data is if bone resorption is already reduced in women with higher VAT, will calcium supplementation, which is the first line of therapy for preventing post-menopausal bone loss, be effective in this group? We hypothesize that there could be interactions between higher VAT and calcium per se and the source of calcium (dietary versus tablet) which could influence CTX-1 suppression. Thus, the primary objective of this study was to evaluate the impact dietary calcium and a calcium carbonate tablet on CTX-1 levels in post-menopausal women with higher and lower VAT.

5.2 Materials and Methods

5.2.1 Participant Recruitment and Screening Visit

Advertisements were placed in free community newspapers, radio and social media encouraging healthy lean and overweight postmenopausal women to participate in a randomized cross-over study investigating the acute effect of two different forms of calcium supplementation on CTX-1 levels. The methodology has already been described in detail in chapter 2 (section 2.2). Briefly, seventy-six apparently healthy normal (BMI < 25 kg/m^2) and overweight (BMI > 27 kg/m^2) post-menopausal women with no osteoporosis, no history of low trauma fractures and no cause of bone loss were recruited for the study.

Ethics approval was obtained from University of South Australia human research ethics committee and the trial was registered as ACTRN12617000779370. All participants underwent body weight measurements, standing height, hip and WC as explained previously in section 2.2.2.1. iDXA was used to measure whole-body fat, lean mass, android fat. VAT was measured using the CoreScan software as explained previously in section 2.2.2.1. Values for total body BMD (g/cm^2), whole body bone mineral content (g), lean mass (g) and fat mass (g) were also obtained. Each participant was randomized using a computer-generated plan to one of the three test products, calcium carbonate tablet (Calsup, two tablets) with 500 ml water providing 1000 mg calcium, high calcium milk 500 ml providing 1000mg calcium (LIDDELLS HI CALCIUM SKIM MILK, 210kcal) or orange juice 500 ml with no calcium providing 210 kcal. Orange juice was used as the control product to understand the effect of calories on CTX-1 suppression. As calories from either carbohydrates, protein or fat is also known to suppress CTX-1 levels [49], we selected orange juice with equal calories to the milk as a control product which allowed us to investigate the additional effect of 1000 mg calcium on CTX-1 suppression over and above the effect of calories.

5.2.2 Intervention visits

All the participants returned to the clinical trial facility after one or two weeks depending on their availability and commitment for three consecutive weeks, one week apart, for 5-hour at each occasion as explained in section 2.2.3. Briefly, the participants arrived at the laboratory after an overnight fast and an indwelling intravenous cannula was placed in an antecubital vein which was kept patent using saline. Baseline serum and plasma specimens were collected, and the participants were given their allocated product. Subsequent serum and plasma specimens were collected at 30 minutes, 1, 2, 3, 4 and 5 hours. The blood specimens were stored on ice and centrifuged at 4000 rpm for 15 minutes within 2 hours of collection. The samples were stored in multiple aliquots at -80^{0}C till further analyses. The participants returned to the laboratory twice after a wash out period of one week each and the same procedure as described above was repeated with a change in the intervention depending on the participant allocation plan.

5.2.3 Assessments

Post-prandial suppression in CTX-1 levels post calcium intervention was the primary outcome measure. Accordingly, plasma samples were analyzed for CTX-1 using fully automatic IDS-iSYS immunodiagnostic assay at baseline, 30minutes,1,2,3,4 and 5-hour time points as explained in section 2.2.4.1. Serum samples were analyzed by chemiluminescent immunoassay (Diasorin LiaisonXL) for 1-84 PTH at baseline,1,2,3,4,5-hour time points as explained in section 2.1.3.6. Serum calcium levels were measured at baseline,1,2,4,5-hour timepoints, phosphorus levels at baseline and at 2-hour time point and ALP levels were measured at baseline, 2-and 5-hour timepoint by Thermo Fisher scientific automated Kone lab analyzer as explained in section 2.2.4.3. All assays were performed as per manufacturer's instructions.

5.2.4 Statistical Analyses

The Shapiro-Wilk test of normality was used to ascertain the normality of the variables. Most of the variables were not normally distributed, hence all values were log transformed. The participants were divided into VAT quartiles and repeated measures of ANOVA was used to investigate the interaction between VAT, calcium intervention and timepoints using VAT quartiles as a-between subject factor. Spearman correlation was used to describe relationships between VAT and CTX-1 & 1-84 PTH levels at all timepoints. The mean maximal suppression of CTX-1 was calculated and expressed as percent change from the baseline value for each of the test product. All the analyses were carried out using SPSS (IBM SPSS Stats 25) and $p < 0.05$ was considered significant.

5.3 Results

5.3.1 Participant Characteristics

Participant characteristics have been described in detail previously in chapter 4. Briefly, the median age of the participants was 65.5 years (IQ range 61.9-68.5 years). 36% women had BMI < 24.9 kg/m^2 and 65% women had BMI > 27 kg/m^2. VAT levels ranged from 91-3392 cm^3 and CTX-1 levels ranged from 138-968 ng/L. The effect of high calcium milk, calcium carbonate tablet and orange juice on CTX-1 and 1-84 PTH levels were studied in the whole cohort and in the different VAT quartiles.

5.3.2 Kinetics of CTX-1 and 1-84 PTH Suppression with Calcium Interventions- Whole Cohort

First, we investigated whether there were any significant differences in the acute effect of 1000 mg milk calcium (210 kcal) and 1000 mg calcium carbonate (0 calories) and juice containing no calcium with 210 kcal on CTX-1 and PTH levels suppression in the whole cohort. All three treatments i.e. juice, milk and calcium carbonate tablet resulted in a significant decline in CTX-1 and PTH levels. Juice resulted in a 12% reduction ($p < 0.000$) in PTH levels from baseline at 2-hour time point but the levels returned to baseline at 3-hour time point. Calcium carbonate tablet resulted in a 46% ($p < 0.000$) reduction in 1-84 PTH levels at 1-hour time point, while milk resulted in a 25% ($p < 0.000$) reduction at 2-hour time point (figure 5.1 A). The PTH levels remained relatively suppressed at subsequent time points for both calcium carbonate tablet and milk ($p < 0.000$).

With regards to suppression in CTX-1 levels, a significant intervention ($p < 0.000$) and time effect ($p < 0.000$) was observed for all three treatments. The degree of suppression in CTX-1 levels was significantly higher with milk and calcium carbonate, around 44% from baseline as compared to juice which resulted in 18% ($p < 0.000$) reduction from baseline (figure 5.1 B, $p < 0.000$). While there was no difference in the degree of CTX-1 suppression between milk and calcium carbonate tablet ($p = 0.478$), the kinetics of CTX-1 suppression was significantly different between these two interventions (figure 5.1 b).

Milk resulted in maximal suppression in plasma CTX-1 levels at 2-hour time point (-44.5%, $p < 0.000$) whereas an equal dose of calcium (1000 mg) as calcium carbonate tablet resulted in maximal CTX-1 level suppression at 5-hour time point (-44.1%, $p < 0.000$). CTX-1 levels remained suppressed at the end of 5-hour with juice ($p < 0.05$), milk ($p < 0.000$) and calcium carbonate ($p < 0.000$).

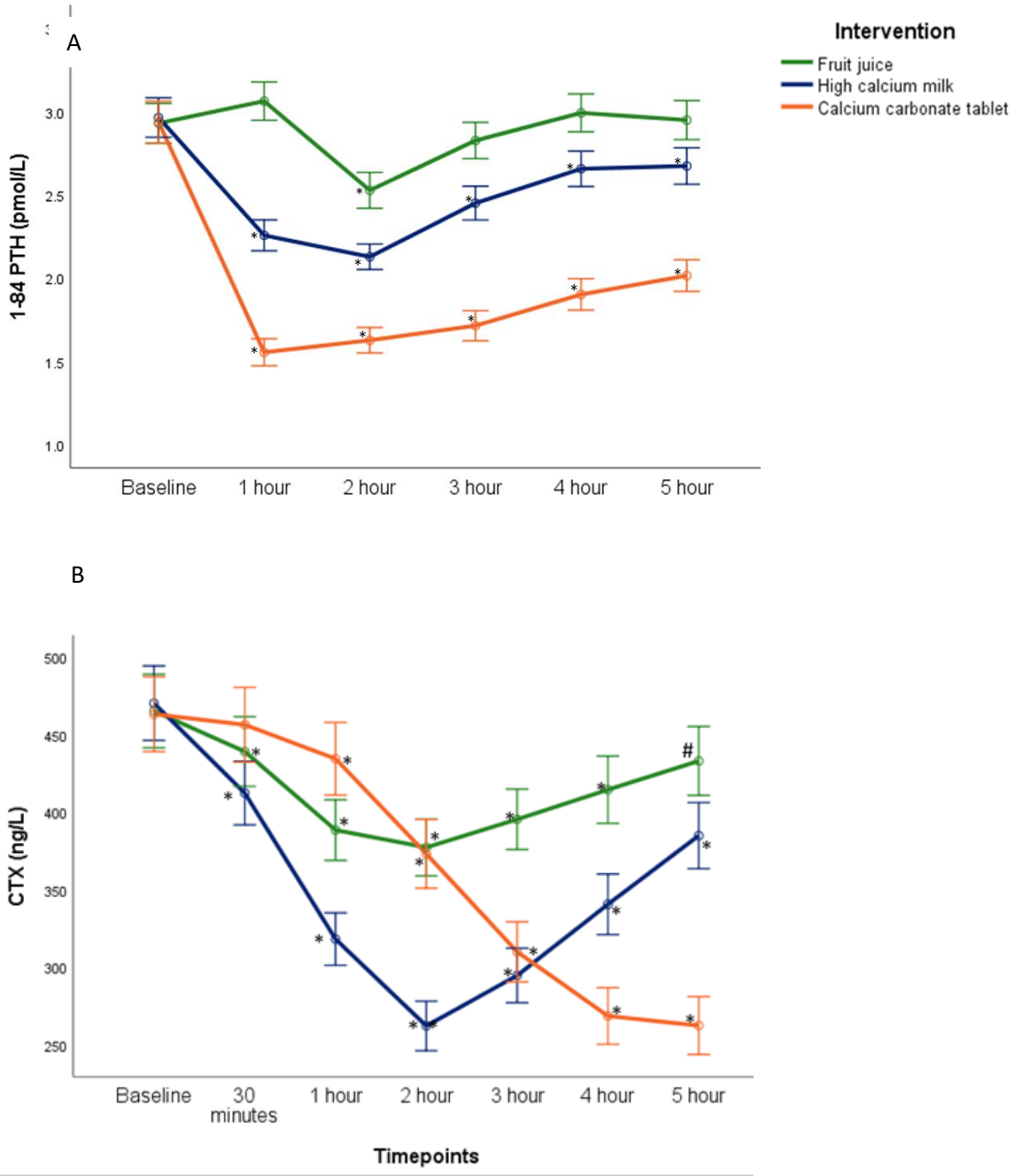

Figure 5. 1 Post-prandial changes in (A) 1-84PTH levels and (B) CTX-1 levels with fruit juice (no calcium, 210 kcal), high calcium milk (1000 mg calcium, 210 kcal) and calcium carbonate tablet (1000 mg calcium, no calories) during 5-hour study period.

Values are means, with their standard errors represented by vertical bars. (*) mean value was significantly different from baseline, $p < 0.000$ (#) mean value was significantly different from baseline, $p < 0.01$.

5.3.3 Kinetics of CTX-1 and 1-84 PTH Suppression after Interventions- Effect of VAT

At baseline, women in the highest VAT quartiles had higher 1-84 PTH levels and lower CTX-1 levels as compared to women in the lowest VAT quartile ($p < 0.05$). Despite these baseline differences, both high calcium milk (figure 5.2 A-B) and calcium carbonate tablet (figure 5.3 A-B) resulted in significant decline in 1-84 PTH levels and CTX-1 levels with no significant between-group differences among VAT quartiles.

Milk resulted in maximum reduction in PTH levels (figure 5.2 A) and CTX-1 levels (figure 5.2 B) at 2-hour time across all VAT quartiles. Calcium carbonate tablet resulted in nadir 1-84 PTH levels (figure 5.3 A) and CTX-1 (figure 5.3 B) levels at 1-hour and 5-hour timepoint, respectively, across VAT quartiles. Interestingly, in the calcium carbonate arm, despite no further decrease in PTH levels after 1-hour time point, CTX-1 levels continued to decline steadily till the end of the experiment (4- and 5-hour time point). Juice resulted in maximal PTH (figure 5.4 A) and CTX-1 levels (figure 5.4 B) suppression at 2-hour time point across VAT quartiles.

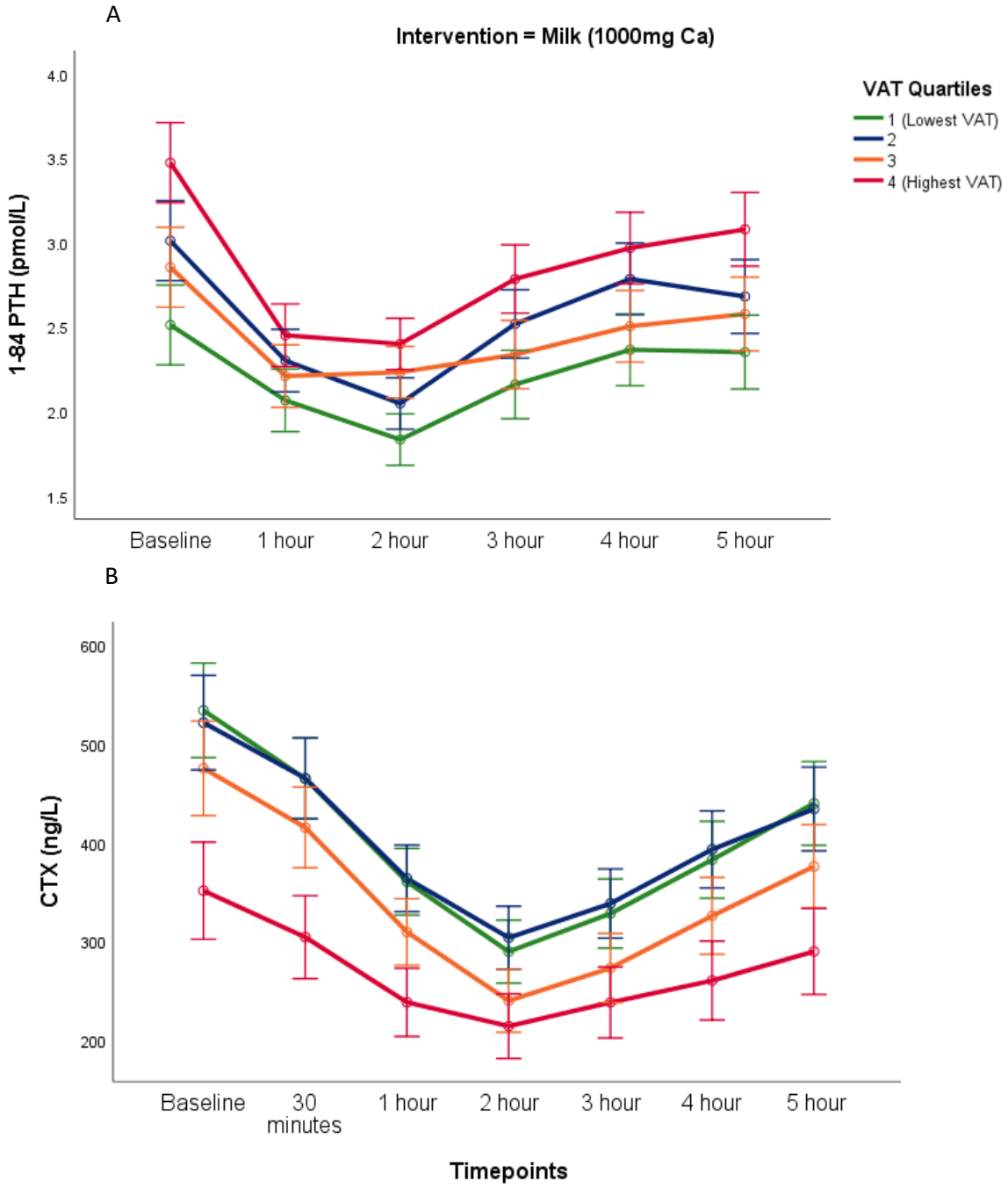

Figure 5. 2 Effect of milk (1000mg calcium, 210 kcal) on post-prandial changes in (A)1-84 PTH levels and (B) CTX-1 levels in post-menopausal women as VAT quartiles during 5-hour study period.

25th VAT quartile (green), 25-50th VAT quartile (blue), 50-75th VAT quartile (orange) and 75-100th VAT quartile (red).

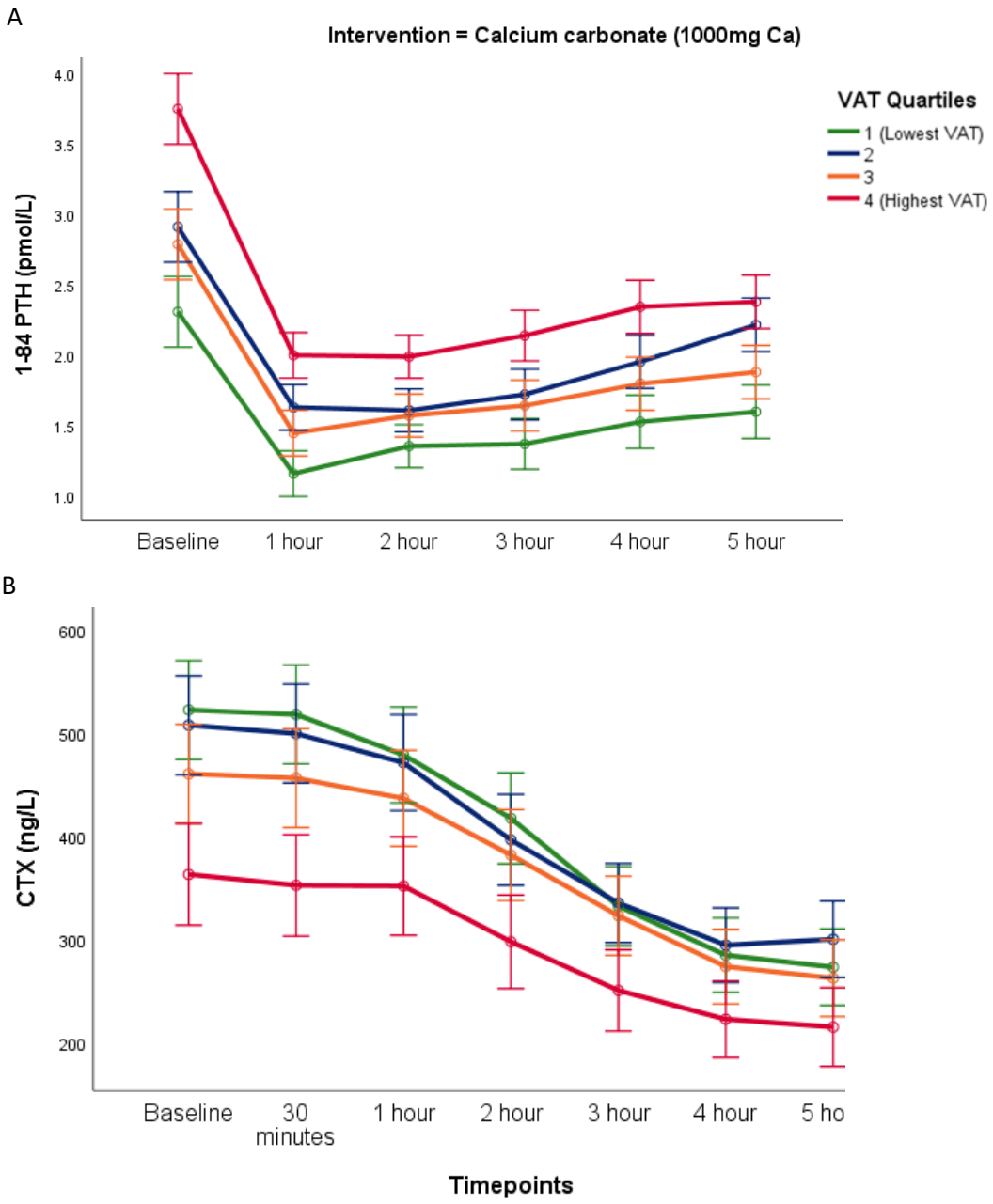

Figure 5. 3 Effect of calcium carbonate (1000 mg calcium, no calories) on post-prandial changes in (a) 1-84 PTH levels and (b) CTX-1 levels in post-menopausal women as VAT quartiles during 5-hour study period.

25th VAT quartile (green), 25-50th VAT quartile (blue), 50-75th VAT quartile (orange) and 75-100th VAT quartile (red)

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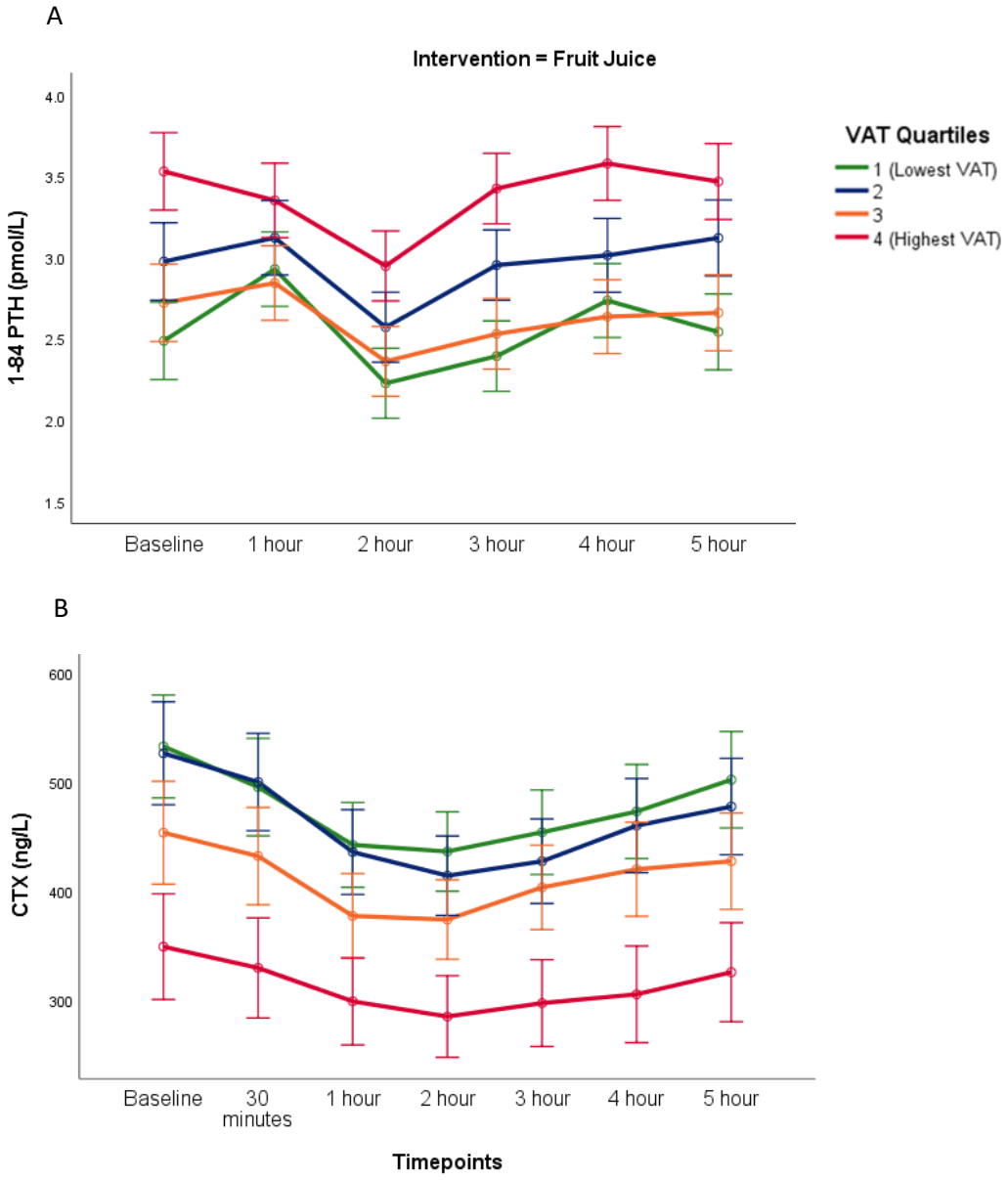

Figure 5. 4 Effect of fruit juice (210 kcal, no calcium) on post-prandial changes in (a) 1-84 PTH levels and (b) CTX-1 levels in post-menopausal women as VAT quartiles during 5-hour study period. 25th VAT quartile (green), 25-50th VAT quartile (blue), 50-75th VAT quartile (orange) and 75-100th VAT quartile (red).

5.3.4 Changes in Serum Calcium, ALP and Phosphorus- Effect of Intervention and VAT

There was no difference in the baseline serum calcium levels across VAT quartiles and the levels increased significantly in response to both milk and calcium carbonate tablet. There was a significant effect of source of calcium i.e. milk calcium vs. calcium carbonate ($p < 0.001$) as well as influence of time on total serum calcium levels ($p < 0.001$). Serum calcium increased in response to milk calcium for in the first 2 hours (2.4% increase) and returned to baseline at 4-hour timepoint. However, after the calcium carbonate intervention, serum calcium continued to increase till 4 hours (5% increase) but plateaued at 5 hours (Fig 5.5). The median calcium levels at 2-hour time point for milk and at 4-hour time point for the tablet were 2.39 mmol/L (IQ 2.33-2.45) and 2.48 mmol/L (IQ 2.42-2.53), respectively. The changes in calcium levels with intervention were independent of VAT levels.

There was no effect of juice and milk on changes in phosphorus levels while the interaction between calcium carbonate tablet and timepoint was significant ($p = 0.001$). However, the magnitude of change in phosphorus levels with calcium carbonate were small, about a 3.4% increase (figure 5.6).

There was no difference between the interventions on changes in ALP levels but the effect of timepoint was significant ($p < 0.000$) and for each of the intervention, the ALP levels were highest at 5-hour time-point ($p < 0.05$) (figure 5.7).

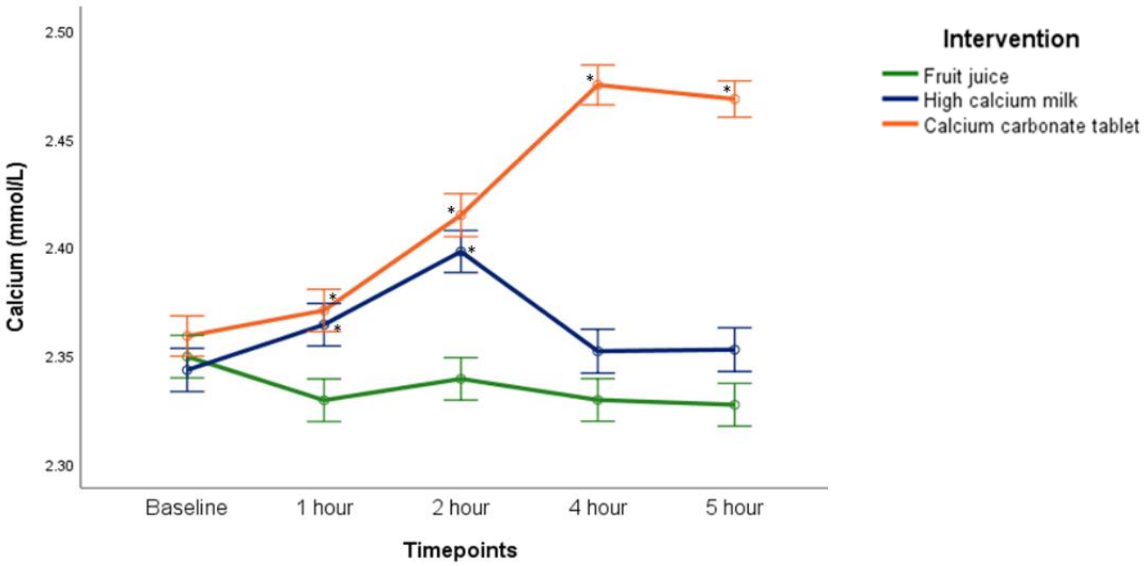

Figure 5. 5 Changes in serum total calcium levels with fruit juice (no calcium), milk (1000 mg calcium) and calcium carbonate tablet (1000 mg calcium) during 5-hour study period.

(*) Mean value was significantly different from baseline, $p < 0.000$.

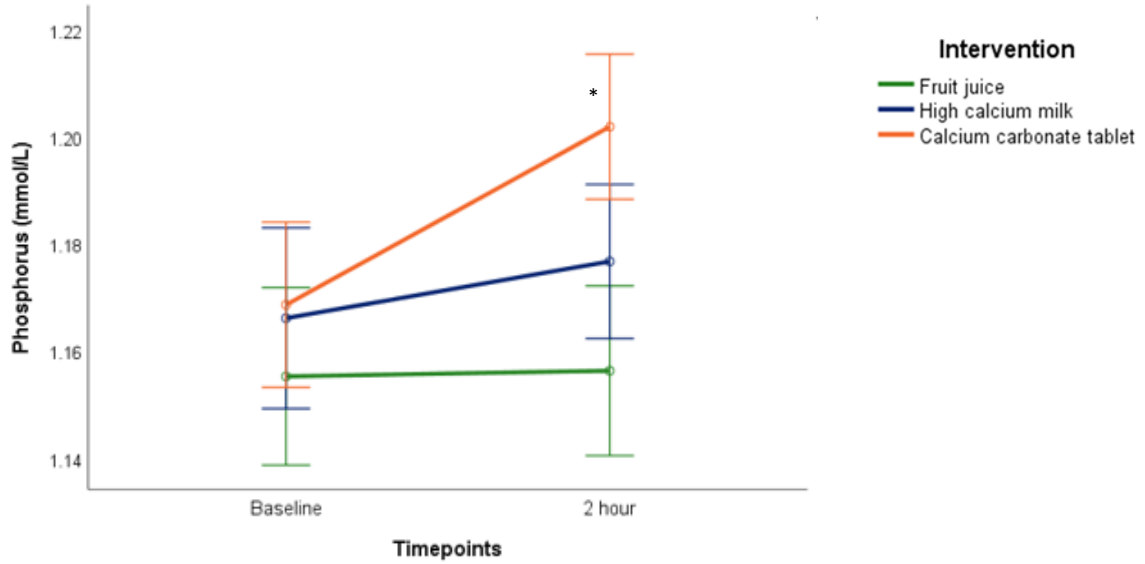

Figure 5. 6 Changes in serum phosphorus levels total calcium levels with fruit juice (no calcium), milk (1000 mg calcium) and calcium carbonate tablet (1000 mg calcium).

(*) Mean value significantly different from baseline, $p < 0.01$.

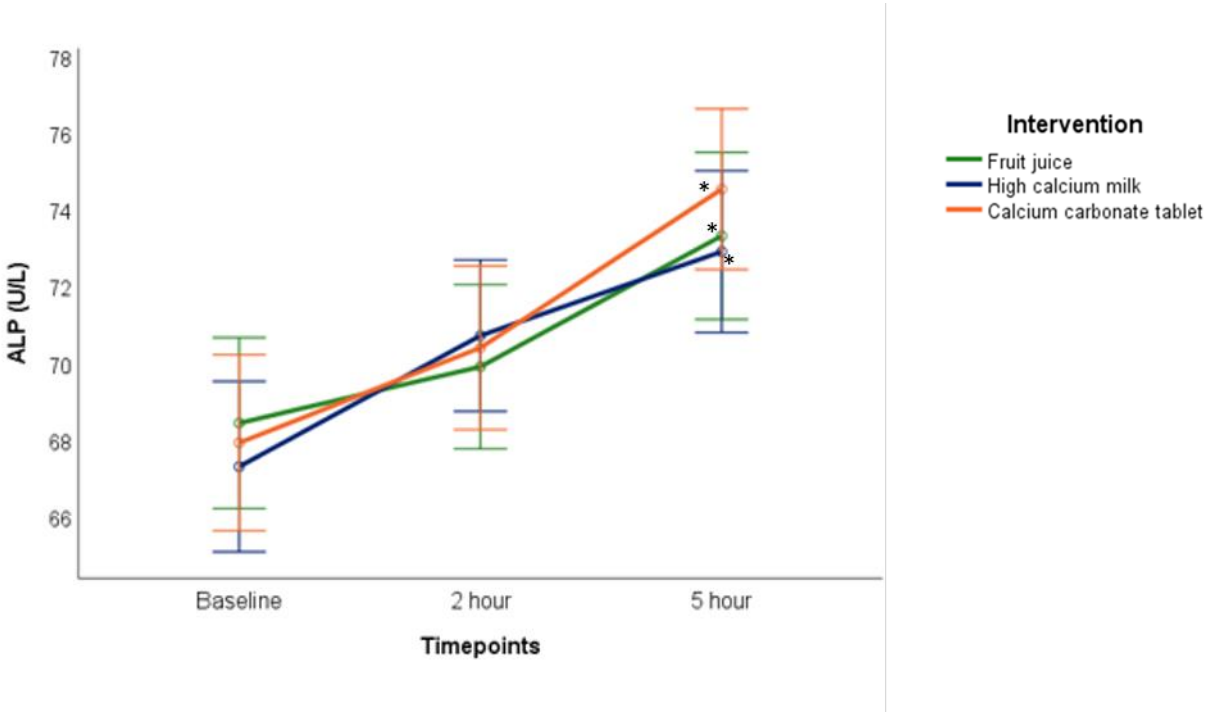

Figure 5. 7 Changes in serum ALP levels with fruit juice (no calcium), milk (1000 mg calcium) and calcium carbonate tablet (1000 mg calcium)

(*) Mean value significantly different from baseline, $p < 0.05$.

5.4 Discussion

We have demonstrated from the first time that higher visceral fat does not alter acute post-prandial suppression in CTX-1 and 1-84 PTH levels in post-menopausal women with milk calcium and calcium carbonate tablet, despite significantly different baseline CTX-1 and PTH levels. The maximal suppression in CTX-1 levels and PTH levels was seen at the 2-hour time point with milk across VAT quartiles. With calcium carbonate, the maximal PTH suppression was seen at 1-hour time point while the CTX-1 nadir was observed at the 4-5-hour timepoint. This is contrast to the findings of Thomas et al.[104] who reported a higher suppression in CTX-1 levels with 560mg calcium in women with WC > 88 cm (n = 16) as compared to women with WC < 88cm (n = 8). However, this was a post-hoc finding and the study was not designed to investigate the effect of WC on acute effects of calcium supplementation. The design of the Thomas et al. [104] study was different from ours (nighttime supplementation and lower dose of calcium) which further makes the

comparison difficult. None of the other studies in the literature have investigated the interactions between calcium intervention and central adiposity and effect on bone resorption. While the effect of VAT on CTX-1 levels and PTH suppression was not significant, there were significant differences in the metabolic responses of milk calcium and calcium carbonate that requires further discussion.

Milk calcium resulted in rapid suppression of PTH and CTX-1 levels, with serum PTH levels and plasma CTX-1 levels nadir occurring at 2-hour, simultaneously with highest total calcium levels. Though PTH and CTX-1 levels begin to gradually rise after 2 hours, the levels at 5-hour were significantly lower than the baseline. The suppression in PTH levels by calcium load is mediated by the CaSR on the parathyroid glands that respond within seconds to flux in the blood calcium levels [487] and several studies have reported this calcium dependent postprandial suppression in PTH levels resulting in concomitant CTX-1 suppression [50-52, 66, 116, 118, 119, 264, 266-269, 271, 488-492].

Calcium carbonate resulted in rapid suppression in 1-84 PTH levels, with nadir PTH levels observed at 1-hour time. Interestingly, despite no further decrease in PTH levels in the calcium carbonate group beyond 1 hour, CTX-1 levels continued to decline progressively throughout the experiment and nadir CTX-1 levels for the tablet were seen at 4-5 hours. These data suggest that post-prandial suppression in CTX-1 levels with calcium carbonate is only partially mediated by suppression in PTH levels and that there are additional mechanisms independent of PTH levels that reduce bone resorption. There is evidence to suggest that CaSR are expressed in osteoclasts and calcium ions may therefore directly regulate bone resorption independent of PTH suppression [493, 494]. Hence, calcium supplementation may directly modulate bone resorption response through activation of CaSR present on the osteoclasts independent of PTH levels. Green et al. compared the acute effects of a reconstituted milk powder fortified with calcium carbonate (1200 mg calcium) and a reconstituted skim milk powder fortified with milk calcium (1200 mg calcium) and demonstrated that both resulted in a maximal CTX-1 levels suppression at 5-hour. The values begin to gradually increase for both the calcium treatments between 5-8 hour. The PTH levels remained virtually unchanged after the milk calcium but decreased

between 1 and 4-hour with milk powder fortified with calcium carbonate [51]. It is hard to compare the findings of our study with that of Green et al., due to differences in the design and the matrix in which calcium carbonate is delivered which may affect the postprandial metabolic responses [259].

The findings of this study also provide indirect evidence on calcium absorption in post-menopausal women from two commercially available sources of calcium which can be easily incorporated in the diet to improve calcium intake. A recent report from the AIHW suggest that 91% of the women in the age range of 51-70 years have calcium intakes less than the EAR [250]. A direct and precise measure of calcium absorption from foods can be achieved by labeling them with radioactive or stable isotopes of calcium [495]. However, these studies are cost intensive, require highly sophisticated analytic capability and testing of commercially available calcium fortified foods or calcium supplements products is difficult using isotopic labelling approaches [496]. Thus, using indirect methods of evaluating calcium-responsive biochemical end-point relevant to bone metabolism such as measurement of serum calcium, PTH levels and CTX-1 levels may be useful to study the effect of calcium on relevant bone biomarkers as reported previously [269, 497-500], though the biochemical indicators of calcium absorption are less sensitive as compared to isotopic tracers. The observed reduction of PTH levels by both milk calcium and calcium carbonate tablet suggests a quick absorption of both the forms of calcium by the active transcellular calcium transport in the duodenum. While the active absorption mainly occurs at lower calcium intake, as the study participants were fasting and had elevated PTH levels, absorption of calcium through the active transcellular pathway from milk and calcium carbonate tablet is plausible. Milk calcium seems to be absorbed mostly through the active process as milk results in maximal rise in blood calcium levels with concomitant reduction in PTH and CTX-1 levels at 2-hour time point. It has been reported that the active calcium absorption is usually completed within 2.5 hours of oral calcium load [501]. On the other hand, calcium from calcium carbonate tablet seems to be absorbed both by active and paracellular processes. Digestion and absorption of calcium from calcium carbonate tablet requires breakdown or dissociation, hence calcium carbonate may be available for longer

time and absorption by active transport or passive diffusion could continue across the length of small intestine leading to a sustained and prolonged PTH and CTX-1 suppression. This is further corroborated by the observation that the maximal blood calcium levels with calcium carbonate are observed at 4-hour time point with a concomitant maximal CTX-1 level suppression between 4-5 hour. Further, there is evidence to suggest that calcium from low molecular weights salts (such as oxalate and carbonate) does not have to be necessarily dissociated to be absorbed [502, 503], hence absorption of intact calcium carbonate complex through paracellular process is also plausible.

Further, differences in gastric emptying may account for some of the kinetic differences between milk calcium and calcium carbonate tablet. Rate of gastric emptying is linearly correlated with calorie density of the ingested meal [504] and the source of the calories i.e. carbohydrates or protein or fat have very little effect [504, 505]. Milk and juice with equal calories (210 kcal) will have a slower gastric emptying as compared to calcium carbonate with no calories which will exit the stomach faster. However, we have not tested this in the current study, and it is a subject for further investigation.

Significantly higher PTH suppression by calcium carbonate as compared to milk observed in our study (47% and 22% respectively; $p < 0.000$) is consistent with previous studies [51, 500, 506] and could be driven by changes in phosphorus levels [507]. It has been reported that hypercalcemia induced suppression of parathyroid function may lead to the increment of serum phosphate which can regulate PTH secretion independent of the concentration of calcium [264]. We found significantly higher levels of phosphate at 2-hour timepoint only after calcium carbonate administration consistent with results of Karp et al., [508] while the levels did not change with milk supplementation.

The effect of calories on bone resorption cannot be negated as supplementation with juice containing no calcium resulted in a significant 18% reduction from baseline ($p < 0.000$) in CTX-1 levels. The equal calories in juice and milk (210 kcal) allowed us to measure the effect of 1000 mg milk calcium over and above the effect of calories in milk derived from protein and carbohydrate. Milk with 1000 mg calcium and 210 kcal resulted in a

significantly higher reduction in CTX-1 levels as compared to juice ($p < 0.000$). The effect of feeding on bone resorption mediated through gut hormones especially glucagon-like peptide (GLP-1 and GLP-2) has been documented in the literature [49, 509, 510] and postprandial suppression in bone resorption is independent of calories only above 250 kcal [511].

Overall, the findings of the present study have important clinical implications. First, as women with different VAT levels respond almost equally to milk calcium and calcium carbonate, we suggest that overweight post-menopausal women will benefit both from milk calcium and calcium carbonate to suppress CTX-1 levels. While our findings are limited to acute effects of calcium on bone resorption, Roger et al. [512] demonstrated using ^{41}Ca tracer methodology that 1000mg of calcium from food (dairy) and calcium carbonate show similar calcium retention. Prince and colleagues showed that milk calcium and calcium carbonate were equivalent in preventing bone loss in postmenopausal women [513]. In contrast, another study reported that while a low-fat milk and calcium carbonate supplement improved calcium balance in 30 healthy postmenopausal women, calcium carbonate suppressed bone remodelling to a greater extent than milk [514]. We observed higher PTH suppression with calcium carbonate but the decrement in CTX-1 levels were similar between the two calcium interventions. The long-term consequences of these changes need to be tested in future studies. Secondly, there is evidence to suggest that weight loss is associated with increased bone resorption [515] and reduction in hip BMD [516]. Based on our findings, we postulate that including calcium as a part of weight loss regimen, may help to attenuate these bone related changes as calcium, both as milk and tablet, is effective in reducing bone resorption independent of adiposity as shown previously by some authors [517, 518]. Third, markers of bone resorption show a circadian rhythm in which they rise overnight and peak early morning [492, 519]. Given that calcium carbonate results in a continuous and sustained decline in CTX-1 levels independent of VAT levels, we add to our previous findings [119] that consuming calcium carbonate at night might be more effective in reducing nocturnal increases in bone resorption levels even in overweight post-menopausal women.

CHAPTER 6: SUMMARY AND CONCLUSION

“Science never solves a problem without creating ten more".

George Bernard Shaw

6.1 Summary

This thesis investigated some of the unresolved questions in the field of vitamin D and calcium and has provided answers to some of the knowledge gaps. The aims were accomplished using a combination of a basic science approach to examine the mechanism by which adequate vitamin D is associated with improved bone structure in an elderly cohort and a randomized cross-over clinical trial to investigate the role of body composition and VAT on bone health in post-menopausal women. The interaction between calcium intake and VAT and whether calcium dosage needs to be adjusted for body size as recommended for vitamin D was also investigated.

Our group pioneered the concept that $1,25(OH)_2D$ synthesized within bone from serum 25(OH)D when accompanied with adequate dietary calcium increases bone mineral status in rodent models [337]. Complementary data have been generated from a series of genetically modified mouse models confirming that bone mineral accrual is positively related to bone cell vitamin D activities independent of changes to circulating $1,25(OH)_2D$ levels, suggesting the possible existence of paracrine-autocrine actions of vitamin D in rodents. The remaining question was whether these activities occur in humans as well.

The first part of this thesis investigated the hypothesis that adequate serum 25(OH)D levels, a prohormone form of vitamin D, may be metabolized locally within the bone resulting in improved bone structure. Intertrochanteric bone biopsies obtained from patients undergoing hip arthroplasty for fractured neck of femur was used as the clinical material. We first investigated the structural differences in trabecular bone quantity and quality variables in this cohort compared to non-fractured controls undergoing elective hip arthroplasty due to hip osteoarthritis . Bone quality differed markedly between the fracture and the non-fracture group. Fracture patients had significantly higher BS/BV and Tb.Pf indicating the increased prevalence of rod-like trabeculae as compared to the higher prevalence of plate-like trabeculae in the non-fracture group. These findings add to the existing evidence that intertrochanteric hip fractures are associated with preferential loss of well-connected plate-like trabecular structure to more disconnected rod-like trabecular

microarchitecture [207]. BV/TV was also significantly lower in the fracture group. The biopsies obtained from the fracture cohort were further investigated to understand if varying serum 25(OH)D levels, relate to micro-structural and histomorphometric measures of bone quality and quantity. The findings showed that higher serum 25(OH)D levels are associated with improved bone formation measured as MWT and presence of plate-like trabecular bone structure evident by reduced BS/BV. These anabolic actions of vitamin D were independent of circulating $1,25(OH)_2D$ levels and PTH levels. Further, the study confirmed that mechanisms associated with trabecular thinning and conversion of plates to rods in hip fracture patients are distinct from changes occurring during the normal ageing process as age was not a determinant of BS/BV in the fracture cohort. Despite these observations all these patients had fractures. Whether the action of local $1,25(OH)_2D$ may have improved the quality of the remaining bone mass and delayed the fractures, is a matter of future studies. No association was observed between vitamin D and any bone quantity variables such as BV/TV and Tb.N. These findings extend the current knowledge on the direct effects of vitamin D on bone health and provide evidence for the independent role of serum 25(OH)D levels in determining bone quality. Furthermore, this study also demonstrated the significance of expression of CYP27B1 gene (vitamin D synthesis) and CYP24A1 gene (vitamin D catabolism) within bone in humans. The results support that adequate vitamin D levels along with higher expression of CYP27B1 and lower expression of CYP24A1 is associated with lower BS/BV consistent with plate-like trabecular structure and provide evidence for the paradigm of local synthesis of $1,25(OH)_2D$ within bone which may result in improved bone microarchitecture and reduce fracture risk. Figure 6.1 shows a proposed mechanism of vitamin D action to improve bone health.

The data generated from this study also support the notion that serum 25(OH)D levels are more strongly associated with micro-structural and bone formation measures as compared to bone quantity. This is in concurrence with clinical data which shows no impact of vitamin D supplementation on bone density measures.

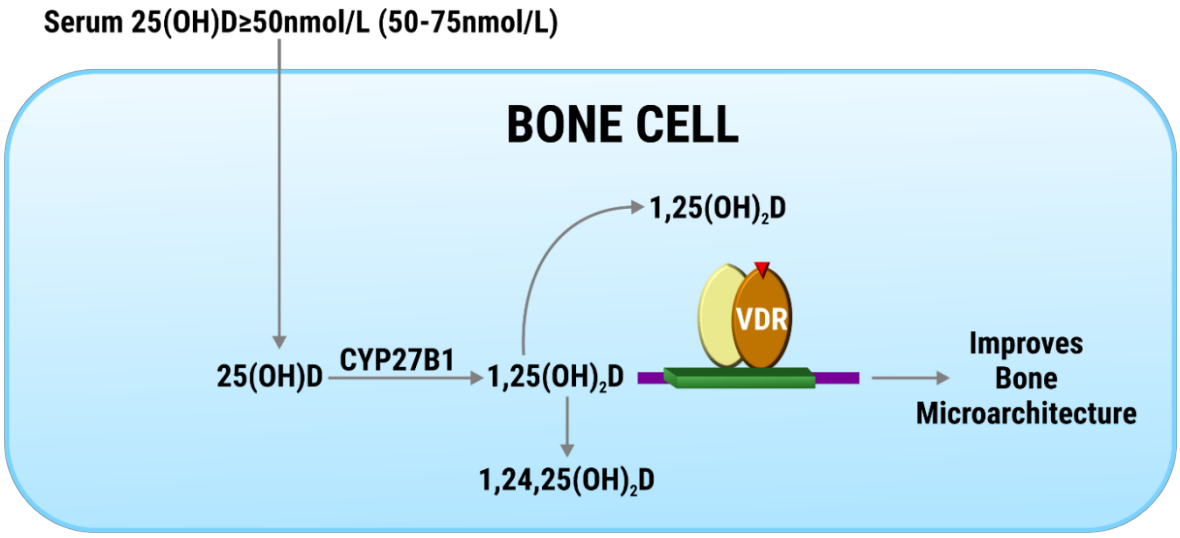

Figure 6. 1 Proposed mechanism for improved bone structure with 25(OH)D levels > 50 nmol/L (50-75 nmol/L)

Further, the data from this study provides some contribution to the clinical debate over adequate levels of vitamin D required for optimal bone health. In this study, vitamin D levels greater than 50 nmol/L were associated with improved bone structure (higher MWT and lower BS/BV). There was a continuous improvement in MWT across vitamin D intervals. Hence, we propose that 25(OH)D levels greater than 50 nmol/L (around 50-75 nmol/L) are required to demonstrate skeletal benefit which is consistent with most of the governmental guidelines [32].

Furthermore, among the patients for whom we had the baseline supplement intake data, the patients who were consuming calcium and vitamin D on admission had significantly lower medullary width, an indicator of bone resorption, and lower trabecular separation. Patients consuming both calcium and vitamin had 10% higher cortical thickness. While this result did not reach statistical significance due to small numbers, a 10% higher cortical thickness may be clinically relevant and emphasize the importance of consuming adequate calcium and vitamin D supplementation to maintain bone health.

The clinically relevant end point of higher vitamin D status is reduction in risk of fracture. This study was not designed to study this endpoint; however, the study may raise questions in the minds of some that all these patients ultimately suffered an osteoporotic

fracture. So, is higher vitamin D status helpful? Well, this study investigated the mechanisms by which higher vitamin D status potentially improves bone health and partly explains the anti-fracture efficacy of vitamin D combined with calcium independent of BMD as reported in clinical studies [20]. Osteoporotic fracture is a multi-factorial disease and can be caused by several factors, including genetic predisposition, age, prior history or family history of fractures, fall characteristics, decreased muscle mass (sarcopenia), visual disturbances, and impaired reflexes. Secondly, we did not have information on long-term calcium and vitamin D for the entire cohort. The study provides evidence that ensuring adequate vitamin D status and dietary calcium intake helps to improve bone quality which may reduce fracture risk in some circumstances. Obtaining bone biopsies from healthy population is extremely difficult in research settings. Hence, trabecular biopsies obtained from a site away from the fracture site is appropriate clinical material to demonstrate the association between adequate vitamin D status and bone quality.

While the current study does not provide a molecular mechanism of how local production of $1,25(OH)_2D$ may result in improved bone microarchitecture observed as reduced BS/BV and higher MWT, previous data generated by our group provides some insights on this aspect. Our group has shown that local supply of $1,25(OH)_2D$ within bone results in induction of Enpp1 mRNA coding for nucleotide pyrophosphatase phosphodiesterase 1 (NPP1) and NPP1 protein levels which promotes hydrolysis of ATP to release pyrophosphate [520]. Pyrophosphate is generally considered an inhibitor of bone mineralization [521], however in the presence of elevated tissue non-specific alkaline phosphatase which is present abundantly in bone, it results in production of inorganic phosphate to stimulate mineralization [520].

Hence, we propose that when 25(OH)D levels are >20 nmol/L, circulating $1,25(OH)_2D$ maintains intestinal calcium absorption through endocrine mechanisms [303] and when 25(OH)D levels are greater than 50 nmol/L (around 50-75 nmol/L), local synthesis of $1,25(OH)_2D$ within bone enhances delivery of inorganic phosphate to mineralization vesicles and promote mineral deposition and improves bone quality. Higher 25(OH)D levels may also results in length of time osteoblast work to deposit bone. A question that may

arise here is if osteoblasts are working for longer in patients with serum 25(OH)D levels greater than 70 nmol/L as compared to patients with the levels between 30-50 nmol/L, then why do the results not translate to improved BV/TV or Tb.Th or Tb.N in these patients? We believe that higher MWT may clinically result in an improved bone quality measure which is not currently measured in this thesis and is an area of further investigation. Another reason could be that dominant osteoclastic bone resorption in the fracture patients significantly exceeds bone formation; hence the net bone volume does not increase.

In summary, this study has demonstrated that higher serum 25(OH)D levels (50-75 nmol/L) enables increased MWT suggesting that osteoblast are able to work for longer. Further, higher levels of vitamin D along with higher expression of bone CYP27B1 and lower expression of CYP24A1 is associated with thicker plate-like structure.

To conclude, findings of this study add to the work done by our laboratory over the past three decades to demonstrate that higher 25(OH)D levels have direct effect on bone structure in humans through local metabolism.

The second part of this thesis investigated the effect of obesity on bone health in post-menopausal women. Traditionally, obesity was thought to be protective for bone health, however, recent studies have challenged this view. Pre-menopausal women with higher trunk fat exhibit reduced bone formation and inferior bone quality despite normal BMD [357] and higher WC has been shown to be associated with higher fracture risk [392]. This study investigated the effect of central adiposity and its individual components – SAT and VAT on bone health in post-menopausal women. Loss of estrogen at menopause is associated with higher bone turnover but this study revealed that obese post-menopausal women exhibit suppressed bone resorption and turnover. This is possibly because obesity is associated with higher circulating estrogen that results from increased aromatization of androstenedione by highly expressed aromatase in adipose tissue [113]. All measures of body fat were positively associated with total body BMD suggesting an apparent positive role of fat including VAT on BMD. However, VAT was a negative predictor of BMD after

controlling for mechanical loading (i.e. BMI) suggesting that higher VAT may be detrimental for bone health. Further, VAT was also a negative predictor for bone resorption and turnover markers. Higher BMD in women with higher BMI was a function of fat content as lean mass was removed in multivariate analyses. The finding that WC (most widely used indicator of VAT in clinical settings) was also negatively associated with OC and CTX-1 levels and that WC was removed from the multivariate model when VAT was entered, suggests that the relationship between WC and osteocalcin and CTX-1 levels could be mediated via VAT and that VAT is a more sensitive and accurate predictor of bone health. WC is a composite of SAT and VAT and may not be truly reflective of relationship between VAT and bone health. We also observed lower 25(OH)D levels and elevated PTH levels in women with higher VAT.

These data suggest that VAT and BTMs may be needed in conjunction with BMI and BMD to predict the at-risk obese population which might be a target group for intervention. The first line of therapy can include adequate vitamin D levels (adjusted for body size) and dietary calcium intake to maintain bone health. We have shown that despite baseline differences in osteocalcin levels, CTX-1 levels, PTH levels and 25(OH)D levels in women with higher VAT, acute doses of calcium (both as supplement or dietary calcium) are effective in suppressing CTX-1 and PTH levels irrespective of VAT levels. Further, given that bone resorption is already reduced in obesity, an important question that might arise is whether suppressing bone resorption will be beneficial for bone health in obesity. In this study, we report a negative correlation between BMD and CTX-1 levels, suggesting that suppressing CTX-1 levels with adequate dietary calcium would be beneficial for bone health. Further, as VAT is an independent negative determinant of BMD, weight loss interventions to reduce abdominal fat (in addition to overall body fat) would be beneficial for bone health. Given that many weight loss interventions are associated with increased bone resorption [515] and reduction in hip BMD [516], including adequate dietary calcium as a part of weight loss regimen may be beneficial in reducing BMD changes [517, 518]. The results of this study indicate that women with higher VAT will benefit equally with dietary calcium or calcium from tablet to suppress CTX-1 and PTH levels. While the suppression in

CTX-1 levels were similar with both calcium treatments, calcium carbonate tablet was effective in reducing PTH levels to a greater degree as compared to milk and the suppression in CTX-1 levels was more gradual and sustained. Hence, based on these findings, we propose that for optimal bone health, post-menopausal women will benefit by including calcium carbonate tablet at nighttime to attenuate nocturnal peak in CTX-1 levels.

Lower bone resorption (lower CTX-1 levels) and turnover (lower osteocalcin levels) with no change in bone ALP, lower 25(OH)D levels and elevated PTH in obese women has several clinical implications and are discussed below.

6.2 Clinical Implications of Reduced Bone Turnover in Obese Post-Menopausal Women

Based on the findings of present study, several mechanisms can be postulated which can explain the paradox of lower bone resorption, higher BMD and yet higher risk of fractures in obese women with higher VAT.

6.2.1 Hyper-mineralized bone

A lower bone resorption possibly is the cause of higher BMD observed in this study and many other studies reported previously. However, a lower bone turnover reflected as reduced osteocalcin levels without any difference in bone ALP levels between women with higher and lower VAT levels and suggest that lower bone turnover may not be coupled to a similar lowering of mineralization (indicated by no difference in bone ALP levels) and the matrix may become hyper mineralized [55, 79]. Hypermineralisation may result in a brittle bone with lesser elasticity which might be one of the reasons for higher fracture risk in obese individuals despite normal or higher BMD compared to lean individuals.

6.2.2 Higher sclerostin levels

Obese post-menopausal women in the present study may potentially have higher

sclerostin levels which is an independent predictor of fracture risk [522]. Sclerostin is a protein secreted by osteocytes and directly influences bone remodelling by inhibiting Wnt pathway [523]. Previous studies have shown that post-menopausal women with higher abdominal fat exhibit higher sclerostin levels [522, 524, 525]. Further, sclerostin was found to be negatively associated with osteocalcin and CTX-1 levels [522, 524, 525]. Additionally, physical inactivity which is common among obese people is associated with higher sclerostin levels [524, 526].

6.2.3 Insulin resistance

Several mechanisms have been proposed that link obesity to IR. Obesity is associated with chronic and low-grade inflammation accompanied with lipid and glucose disorders, which is partially caused by macrophages infiltration into adipose tissue [527]. It is also suggested that adipose tissue hypertrophy increases the risk of IR, due to improper deposition of triglycerides in tissues not suited for lipid storage which induces cellular dysfunction. Of the several lipids that accrue under these conditions, sphingolipids such as ceramide or its metabolites are amongst the most deleterious because they disrupt insulin sensitivity, pancreatic β cell function, vascular reactivity, and mitochondrial metabolism [528]. The mechanisms through which ceramides causes insulin resistance include inhibition of insulin and growth factor signaling and action, impairment of mitochondrial lipid oxidation, oxidative stress, and induction of apoptosis [528]. In addition, adipocyte hypertrophy associated with obesity may cause insulin resistance [529]. Hypertrophic adipocytes are also a source of proinflammatory cytokines that may cause insulin resistance [530]. Further, obesity may also result in IR due to energy surplus in some cases. Excess ATP may induce IR by inhibiting AMPK signaling pathways [531]. This is further supported by the observation of improved insulin sensitivity in response to caloric restriction and weight loss [531]**.** Central adiposity is also linked to insulin resistance. Visceral adipocytes have higher lipolytic activity than other fat cells resulting in elevated portal free fatty acid levels which can induce IR through its action on the pancreas, liver and muscle. Free fatty acids may increase pancreatic insulin secretion (but also may reduce

it), decrease insulin sensitivity in muscle and liver, increase hepatic VLDL secretion and induce endothelial dysfunction [350].

Another mechanism through which obese people might be predisposed to developing IR is through link between osteocalcin and energy metabolism. The present study demonstrates that women with higher VAT have reduced osteocalcin levels. Other studies have shown that serum osteocalcin concentrations are inversely associated with fasting plasma glucose, insulin and homeostasis model assessment (HOMA) for insulin resistance [532, 533]. Studies have also shown that obese volunteers with suppressed CTX-1 and OC levels have fasting insulin levels twice that of lean sex and age matched control volunteers an effect mediated via visceral adiposity independent of BMI and total fat mass [465, 466]. Further, data in rodent models show that mice lacking osteocalcin encoding gene exhibit higher VAT, glucose intolerance and impaired insulin secretion compared with wild-type mice suggesting that reduced osteocalcin levels may cause VAT accumulation [534]. Thus, there appears to be a two-way interaction between osteocalcin and VAT levels in causation of IR. Lower osteocalcin levels may cause VAT accumulation resulting in insulin resistance or higher VAT may cause reduced osteocalcin levels which in turn leads to insulin resistance.

Insulin resistance has been associated with reduced bone strength and is an independent risk factor for fracture risk in otherwise healthy obese (non-diabetic) post-menopausal women [467].

6.2.4 Inflammation

Higher VAT results in higher expression of proinflammatory cytokines such as IL-6, IL-8 and TNF-α and lower expression of adiponectin which is potentially protective for bone health [114]. Adiponectin receptors have been shown to be down regulated in obesity linked insulin resistance [535, 536]. Some studies have reported that lower adiponectin levels are a risk factor for fracture [537-539] although results have not been consistent [540].

6.2.5 Elevated FGF-23

Higher VAT has been reported to be associated with elevated FGF-23 levels in a cross-sectional cohort of middle-aged men and women [541]. FGF-23 is an endocrine hormone that regulates phosphate and vitamin D metabolism and higher FGF-23 levels are associated with phosphate wasting resulting in poor bone mineralization [542]. Though the serum phosphorus did not differ across the VAT quartile in our study, a negative correlation between phosphorus and VAT levels was observed ($r = -0.235$, $p < 0.05$). High FGF-23 levels are associated with impaired trabecular bone microarchitecture in osteoporotic patients [542]. Hence women with higher VAT may have elevated FGF-23 which may be another plausible reason for poor bone microarchitecture in obese population.

Overall, findings from this study provides a conceptual framework for understanding the paradox of higher fracture risk in obese individuals despite higher BMD. Higher body fat increases the proportion of bone mass and thus BMD at all sites. However, the impact forces in a fall are higher in obese individuals and higher BMD may not be compensate for the increase in fall impact [388, 390]. Further, a reduction in bone strength resulting from several mechanisms as summarized in figure 6.2 may offset the protection conferred by increased soft tissue padding. The balance between these factors determines the overall fracture risk in an individual. Further, the magnitude of these competing forces can vary between different body sites, hence the relationship between obesity and fracture risk is site-specific as reported in the literature. This study adds to the existing knowledge and suggests that fracture risk may also be influenced by the amount of VAT and its resulting metabolic consequences.

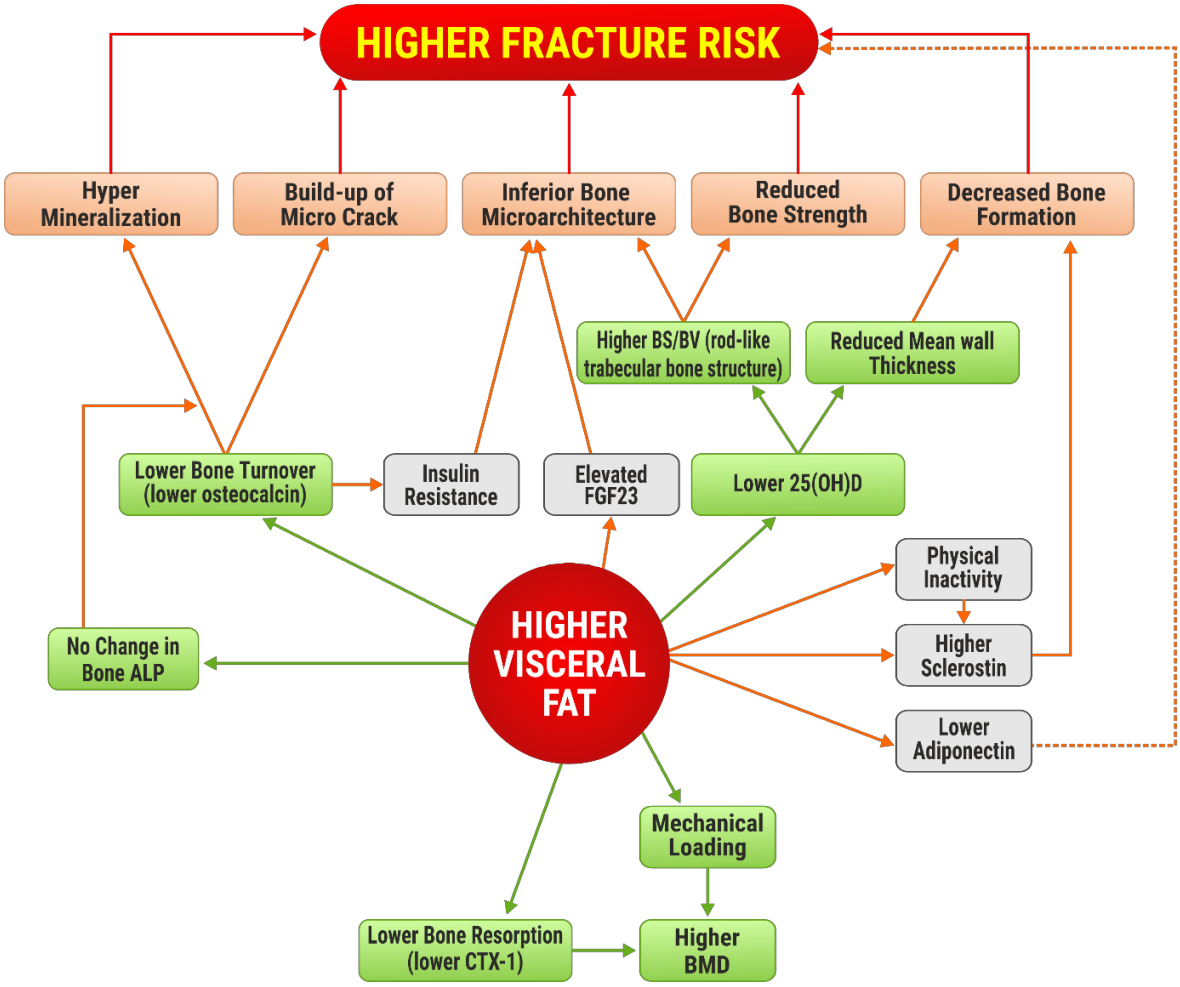

Figure 6. 2 Conceptual framework for the effect of obesity on bone health- the balance between these factors determines the overall fracture risk in an individual

The green arrows (and boxes) depict mechanism demonstrated in this thesis while orange arrows (and grey-boxes) depict proposed mechanism (hypothesis). Dashed line indicates inconsistent evidence.

6.3 Strengths

This is the amongst the first studies in human bone to demonstrate the existence of paracrine-autocrine vitamin D activity. By studying the cellular and molecular mechanism of vitamin D on bone health, we have been able to propose two novel measures that are associated with vitamin D. RCTs of vitamin D supplementation have shown minimal impact when BMD has been investigated as the outcome measure. The findings of this study suggest that including bone quality measures as a part of clinical trials investigating the effects of vitamin D may help to reveal positive effects of vitamin D on bone health. This is the first study to identify the association between vitamin D and two novel measures of bone quality – MWT and BS/BV.

Also, we have reported for the first time the association between VAT and bone remodelling markers in obese post-menopausal women. Previous studies have shown the effect of central adiposity on bone remodelling markers in young men and premenopausal women. This study adds data on another cohort, post-menopausal women are at risk for age related loss of bone mass and demonstrate that obese postmenopausal women remain in a suppressed state of bone turnover even after menopause. By studying women with varying VAT levels, we have been able to propose possible mechanisms by which VAT may exert detrimental effect on bone microarchitecture and strength despite higher BMD, thus predisposing individuals to a higher fracture risk. Further, measurement of VAT by using iDXA allowed us to segregate the effects of abdominal VAT from SAT. Previously, it has been shown that vitamin D therapy needs to be adjusted for body size. To the best of our knowledge, up until now, none of the studies have investigated if dietary calcium levels need to be adjusted for body size and if there is a difference between the actions of dietary calcium versus. calcium supplements in reducing PTH levels and CTX-1 levels, which are calcium responsive clinically relevant end points in post-menopausal women with higher VAT levels. All the previous studies have included lean women and the interactions between

BMI/central VAT and bone remodelling markers and calcium supplementation have not been investigated. We report that irrespective of BMI or VAT levels, all women respond equally well to 1000 mg of dietary calcium as well as 1000 mg calcium carbonate.

6.4 Limitations

I. The studies carried out in this thesis are cross-sectional in design; hence causality cannot be proven.

II. The data obtained from fracture patients provides evidence for local metabolism of vitamin D within bone cells. However, confirmatory evidence would be obtained by measuring $1,25(OH)_2D$ levels within bone cells.

III. In the study on fractured cohort, we could not get data on baseline dietary calcium intake and the baseline supplement intake data was also not available for the entire cohort. Hence, it is difficult to conclusively say if the microstructural differences observed in patients with higher vitamin D status is a function of both vitamin D and calcium intake. However, whether in this elderly cohort, with median age 86 years, it is reasonable to assume that patients with higher vitamin D status (greater than 50 nmol/L) had calcium intakes in the range of EAR remains to be determined. Further, dietary studies conducted by our group in animals show that both adequate calcium and vitamin D are required to improve bone structure through local metabolism [342].

IV. In the clinical study, lack of PINP data (recommended bone formation marker by the IFCC-IOF WG-BMA) limits our ability to conclude on the association between VAT and bone formation in post-menopausal.

V. The precision of iDXA to assess VAT improves with increasing BMI and VAT levels and % CV is higher for BMI < 25 kg/m^2 [52]. However, most of our findings are based on women with higher VAT levels.

VI. We did not measure site-specific BMD. It would be more informative to generate site-specific BMD data in post-menopausal women with higher VAT levels.

6.5 Future Directions

The findings of the cross-sectional evidence generated as a part of this thesis have raised many further questions and paves the way for future prospective studies. Some of the areas that warrant further investigation and will help to inform clinical practice are briefly discussed below.

1. We propose that local metabolism of 25(OH)D to $1,25(OH)_2D$ within the bone helps to improve bone microarchitecture. A further study to investigate the amount of $1,25(OH)_2D$ levels in the bones of young and elderly people with varying 25(OH)D levels and assessing bone microarchitecture by micro CT would be helpful. Along with this, measurement of metabolic endpoints such as calciotropic hormones and bone remodelling markers in young and elderly people will help to establish proof for the proposed hypothesis (Fig 6.1). In addition, histomorphometric analyses to measure the life span of osteoblast such as TUNEL stain would confirm if osteoblast apoptosis is reduced with higher levels of 25(OH)D. Such a study could be done using cadaver bone specimens for the younger cohort and bone biopsies obtained from patients undergoing hip arthroplasty for the elderly cohort. Outcomes of the proposed study would help to identify optimal 25(OH)D levels required for bone health and exploring serum 25(OH)D levels as one of the future biomarkers of bone quality and potentially fracture risk.

2. More important than performing further meta-analyses and systematic reviews on vitamin D and calcium, the findings of this study propose that a statistically powered RCT of calcium and vitamin D supplementation in community dwelling older men and women investigating the effects of supplementation on bone quality using HR-pQCT, vertebral bone quality

using trabecular bone score assessed by iDXA and potential biomarkers would inform the field significantly. Since this study began, some of the researchers have published evidence of effects of vitamin D and/or calcium on bone quality measures. In 2017, Paschalis et al.[445] showed in a retrospective analysis in post-menopausal osteoporotic women, who were a part of the placebo group in two antiresorptive trials and received 400-1200 IU vitamin D and 1-1.5 g calcium supplementation, that supplementation with these nutrients was associated with improved bone matrix properties with higher pyridinoline content and lower glycosaminoglycan content and mineral/matrix at actively forming trabecular surfaces assessed by Raman spectroscopy. Further, Bislev et al.[444] published the results of a 3-month RCT of 2800 IU vitamin D supplementation on bone quality measures and BTMs in eighty one post-menopausal women and demonstrated that vitamin D supplementation for three months improved bone strength and trabecular thickness in tibia as assessed by HR-pQCT and volumetric BMD measured by QCT at the trochanter and femoral neck without any changes in aBMD. No effect on bone remodelling markers was observed. This study was conducted in a Danish population whose baseline calcium intake was around 800mg in the placebo as well as the intervention group. The baseline vitamin D levels were 33 nmol/L which increased to 60 nmol/L at the end of 3 months. We propose a statistically powered study with longer duration or dosage adjusted trial which can increase 25(OH)D levels up to 70-75 nmol/L along with 1000-1300 mg calcium assessing bone quality measured using HR-pQCT and TBS as a primary outcome would help to demonstrate the benefits of vitamin D and calcium on bone quality. Long term follow-up with fracture incidence as another outcome would be highly informative. The study should consider baseline calcium intake and 25(OH)D levels and the population with inadequate calcium and vitamin D intake should be selected with at least 80% compliance. This is because both calcium and vitamin D are threshold

nutrients and the effect would be observed when there is a deficiency and only when the nutrients are consumed.

3. The findings of this thesis also open an altogether new paradigm of improving of bone quality by targeting the bone supply of $1,25(OH)_2D$. From a drug development perspective, novel molecules that can inhibit bone expression of CYP24A1 may be of potential interest for future research. This work is being pursued by our laboratory currently.

4. The interaction between obesity and bone remodelling markers observed in this study suggests that future mechanistic studies in post-menopausal women investigating the role of novel biomarkers such as sclerostin, FGF-23, adiponectin and their associations with bone remodelling markers and bone microarchitecture in women with low and high VAT would help to explain the possible links between obesity, poor bone microarchitecture and higher fracture risk. The findings of the proposed study could also help to identify a potential biomarker for bone health in obese population.

5. A weight loss study including bone related outcomes such as bone quality and density, bone remodelling markers, vitamin D levels with and without calcium and vitamin D supplementation would help to confirm if there is benefit of including these nutrients as a part of weight loss program in post-menopausal women as proposed in this thesis.

6. This study did not include the aspect of bone marrow fat on bone remodelling markers or BMD. Future studies exploring the role of marrow adiposity and its association with VAT and effect on BMD and bone microarchitecture would help to further clarify the role of marrow adiposity on bone health obese individuals.

7. In this study, we observed lower 25(OH)D levels in women with higher VAT.

The plausible reason proposed for lower 25(OH)D levels in obese individuals in the literature is volumetric dilution and fat sequestration. However, none of the studies so far have investigated if the catabolism of vitamin D is higher in obese individuals which could be one of the reasons for lower 25(OH)D levels in obese women. Measuring 24,25(OH)2D levels in populations with varying BMI or VAT levels will shed some light on this crucial aspect. Further, we did not observe any association between 25(OH)D levels and BMD or bone markers in this study. However, it is possible that lower 25(OH)D levels in obese people may affect muscle function adversely. Further studies comparing the effect of vitamin D status and muscle function, gait and fall propensity in people with varying VAT amounts may help to inform preventative strategies for fall prevention in obese individuals which is a major risk factor for fractures in obese people.

8. The effects of elevated PTH levels in obese population has not been clarified up until now. Lower 25(OH)D levels in women with higher VAT explains a part of the story. But the fact that VAT independently accounted for 13% variability in PTH levels suggest that VAT may have direct influence on PTH levels. A possible explanation on the links between VAT-PTH may arise from the fact that VAT secrete higher level of proinflammatory cytokines such as IL-6, IL-8, CRP [350]. Whether any of these factors influence PTH secretion in obesity requires further investigation.

9. It has been reported that leptin levels influence PTH and FGF-23 levels through endocrine and paracrine mechanism [543]. We believe that investigating the relationship between FGF-23, leptin and PTH levels in individuals with varying levels of VAT would be worthwhile to explore. The outcomes of the study may provide insights on reasons for elevated PTH in obesity and will throw further light on the complex fat-bone association. Further, it has been reported that duration of adiposity may also affect PTH

levels [544]. We have not investigated the duration of adiposity in our study and future studies including this aspect may be useful.

10. A recent genetic association study in men and women (age 65-101 years) followed up for 26 months showed that functional variation within the *CYP27B1* promoter gene is associated with higher fracture risk [545]. Further, a recent 30-month vitamin D supplementation (800 IU vitamin D) trial in type 2 diabetic people have demonstrated that serum 25(OH)D level in patients with diabetes mellitus is associated with CYP27B1 polymorphism. Even after long term supplementation, there were still about 50% of the patients whose 25(OH)D levels did not reach 75 nmol/L suggesting that genetic polymorphism affects patient's response to adequate vitamin D supplementation [546]. Type 2 diabetic people, like obese people, present with higher BMD but the risk of fracture is not low in this group [79]. Higher fracture risk in diabetic population is partly linked with microarchitectural alterations in the bone. As CYP27B1 is a vitamin D responsive gene, we propose that a future study in a diabetic population involving vitamin D supplementation may be clinically relevant. The study should include single nucleotide polymorphism analyses to ascertain vitamin D dosage required to raise serum 25(OH)D levels to 75 nmol/L and the outcome measures should be changes in bone quality using HR-pQCT and TBS.

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Appendix I Participant Information Sheet

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Participant Information Sheet

Acute effect of calcium carbonate vs milk on Bone resorption marker (CTX-1)

1. Introduction

You are being invited to take part in a study of the relationship between dietary calcium intake and bone health. This Participant Information Sheet tells you about the research project. It explains the purpose of the research, procedures and risks involved. It also describes information from you that will be collected, how that information will be used and with whom it will be shared. Knowing what is involved will help you decide if you want to take part in the research. Please read this information carefully. Ask questions about anything that you don't understand or want to know more about. Before deciding whether or not to take part, you might want to talk about it with a relative, friend or your local doctor.

If you decide you want to take part in the research project, you will be asked to sign the consent section. By signing it, you are telling us that you:

- Understand what you have read
- Consent to take part in the research project
- Consent to have the tests that are described
- Consent to the use of your personal and health information as described.

You will be given a copy of this Participant Information Sheet to keep.

2. What is the purpose of this research?

The purpose of this research are to investigate whether supplementation with dietary calcium can reduce blood markers of bone breakdown and improve bone health in overweight postmenopausal women to the same degree as calcium supplements do in women of normal weight. Previous studies have shown that adequate calcium nutrition plays an important role in reducing age-related bone loss (primarily occurring after menopause) which if uncontrolled can ultimately result in osteoporosis,. Hence, calcium supplementation is an effective preventive strategy to maintain bone health in post-menopausal women. However, so far all the studies assessing the impact of calcium

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supplementation on bone health have been done in lean or thin women. Hence, there is a need to conduct a well-designed randomized study to assess the impact of calcium supplementation on bone resorption marker (CTX) in overweight post-menopausal women.
Osteoporosis and overweight both impose major cost burdens for their associated care. There is increasing evidence of a high prevalence of fractures in overweight and obese people. We propose to investigate two forms of dietary calcium, a high calcium milk and calcium carbonate supplement to see if they have different effects on bone health in overweight post-menopausal women.

The results of this research will be used by the study investigator Deepti Katyal Sharma, to obtain a Higher Degree by Research, Doctorate of Philosophy. This study is under the principal supervision of Professor Howard Morris, Chemical Directorate, SA Pathology and Professor at University of South Australia and the co-supervisory team, Professor Peter Clifton and A/Prof. Paul Anderson from the University of South Australia.

3. Do I have to take part in this research project?

This is a research project and you do not have to be involved. Further, you are free to withdraw from the project at any time after you have commenced.

4. Who is eligible for the study?

This study is open to healthy lean (BMI less than 25 kg/m^2) and overweight (BMI more than 27 kg/m^2) post-menopausal women (at least 5 years post menopause). If you don't know your BMI, please come for the screening and will confirm your BMI.

In this study, we are unable to recruit post-menopausal women who have been diagnosed with osteoporosis and are on any kind of bisphosphonate therapy or other medications known to affect bone (within the previous 6 months), ongoing conditions known to cause abnormalities of calcium metabolism or skeletal health, malabsorption syndromes (such as coeliac or Crohn's disease or lactose intolerance), diabetes, fracture in the past 12 months, chronic kidney disease, alcoholism. Further, you must agree to stop the use of any health food drinks/beverages and calcium or vitamin D supplements, including herbal supplements at least 2 days prior to the visit 2, 3 and 4th visit as they may influence the study results.

5. What does participation in this research involve?

If you are interested in [participating in this study, you will be requested to attend the clinical trial facility at University of South Australia on four different occasions.

During the first visit, which is a screening appointment, study investigators will

1. Explain the project and you will have the opportunity to ask questions and then sign a consent form should you choose to participate in the study. You are welcome to have a family member or friend present while the project is explained.

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2. You will be requested to provide information about your medical history and any current medications you may be taking. It is important to tell the research staff about all treatments or medications you may be taking including vitamins or herbal remedies.

On visit 2nd, 3rd and 4th visit, you will have to come between 8.00-8.15am to the clinic before eating anything but you can drink as much water as you wish. Each visit will be separated by 1 week. On each of the test days, blood samples will be collected at the start of study followed by intake of one of three test drinks in random order. Blood samples (2ml) will be collected subsequently at 30 min, 1, 2, 3, 4 and 5 hour time points. To facilitate repeated blood sampling, we will request your permission to insert an intravenous cannula for repeated blood sampling. The three products will be a:

1. 500mLHigh calcium milk to provide 1000mg calcium
2. Two tablets of a calcium supplement (CALSUP) with 500mL water providing 1000mg calcium.
3. 500mL no added sugar orange juice

We will also assess your fat mass, muscle mass and bone density using a state of art imaging technique (Dual-X-ray Absorptiometry, DXA) at one the visits depending on your convenience.

The specific details of what will occur at each visit is provided below in a table below.

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Table 1: Timetable of clinic visits, time commitments & procedures performed

Study Stages	Location	What you need to do / what will happen	Time needed
Visit 1 : Screening visit	Clinical Trial Facility, University of South Australia, City East campus	If after reading the study details & considering your options you decide to sign up, the following will be required: - Filling out of the consent form -Measurement of height, weight, hip and waist circumference -Filling out the Trial Entry Form -Body composition assessment using DXA	1 hour
Visit 2	Clinical Trial Facility, University of south Australia, City East campus	-Insertion of intravenous cannula -Collection of 1st blood sample -Consumption of test drink 1 -Collection of subsequent blood samples after 30min,1,2,3,4 and 5 hr	~5 hours
1 week break			
Visit 3	Clinical Trial Facility, University of south Australia, City East campus	-Insertion of intravenous cannula -Collection of 1st blood sample -Consumption of test drink 2 -Collection of subsequent blood samples after 30min, 1,2,3,4 and 5 hr	~5 hours
1 week break			
Visit 4	Clinical Trial Facility, University of south Australia, City East campus	-Insertion of intravenous cannula -Collection of 1st blood sample -Consumption of test drink 3 -Collection of subsequent blood samples after 30min, 1,2,3,4 and 5 hr	~5 hours

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6. Will taking part in this study cost me anything, and will I be paid?

There are no additional costs associated with participating in this research project. All the products will be provided to you free of charge. Further, upon successful completion of the study, you will be paid an honorarium of $200 for your time.

7. What are the possible benefits of taking part?

There is no direct benefit to you, however your participation will help us to find out which form of calcium supplementation is better to improve bone health. This is an important study because calcium supplementation, though advocated widely, is infrequently practised. Further, understanding of the particular form of calcium supplementation will help to inform nutritional guidelines to maintain bone health in lean and overweight post-menopausal women.

8. What are the possible risks and disadvantages of taking part?

All medical procedures involve some risk of injury. We do not foresee any risks to the participants. Cannulas will be inserted by trained professional but there could be slight discomfort during their insertion such as a sharp sensation, which may last for a second or two. Further, in some participants, cannula insertion may result in slight bruising and appropriate treatment would be done to facilitate healing. The broken skin area will be covered with a waterproof dressing. All needles are sterile and are used only once. All needles will be disposed into a sharps-container. The area of needle insertion will be sterilised with an alcohol swab prior to insertion.

9. Can I have other treatments during this research project?

Participation in this study will have no impact on your on-going treatment. You can continue your usual treatment and medicines during the course of this study.

10. What will happen to information about me? Confidentiality and data security

Each participant will be assigned a code known only to the investigators, and this code will be used throughout the analysis and reporting of results from this study. All information will be stored in password protected files and in buildings that are locked after hours.

All your data will be analysed and discussed in coded format. Further, any publication arising from this study will have no specific information regarding you and all data will be anonymous. In addition to the processes described above, the data collected as a part of this study may otherwise be discoverable through processes of law or for assessing compliance with research procedures. You have a right to access the information collected and stored by researchers about you. You also have a right to request that any information with which you disagree be corrected. You have a right to ask that any stored specimens be destroyed but should be aware that data which has already been derived from those specimens may not be able to be destroyed. All the information collected will be retained for 15 years as per Australian research retention guidelines. If any participant presents with an abnormal value for CTX, the study investigators may refer to their GP for further medical follow-up, if you consent.

Research and Innovation Services
UniSA
Human Research Ethics Committee
PARTICIPANT INFORMATION SHEET

Further, upon completion of the study, you will be provided a printed copy of your DXA report which provides information on fat, muscle mass and bone density.

11. What will happen to my test samples?

You will be asked to provide consent for the collection of your blood and few body measurements for this study. Blood samples will be collected by trained personal and will be analysed at SA Pathology. All samples collected will be coded but re-identifiable if required. Privacy/confidentiality of stored tissue samples will be maintained. Any sample remaining after completion of the study will be securely stored in a freezer. After a certain time, samples will begin to degrade and need to be destroyed and appropriately disposed of.

12. What happens if I withdraw from the research?

If you withdraw from the study after 1 or 2 visits, your data will not be included in the study. Further, this is a research project and you do not have to be involved. You are free to withdraw from the project at any time after you have commenced.

13. What happens if I am injured during taking part in the study?

We do not foresee any risk of injury arising from this study. In the event of an injury which is directly related to the study, care would be provided through the public hospital system. The study has been indemnified by UniSA but you have the right to seek compensation through the legal system. Further, your participation in this study shall not affect any other right to compensation you may have under common law.

If you are interested in participating or would like more information about the study please contact **Ph.:8302 1365 or email:** sansom.researchvolunteers@unisa.edu.au

This project has been reviewed by the University of South Australia's Human Research Ethics Committee. If you have any ethical concerns about research or questions about your rights as a participants please contact the Executive Officer of this Committee"

Ms. Vicki Allen;
Tel: (08)83023118
Email: vicki.allen@unisa.edu.au"

Appendix II Participant Consent Form

University of South Australia | Sansom Institute for Health Research

PARTICIPANT CONSENT FORM (UniSA Ethics no: 36365)

1. I, ...*(please print name)* consent to take part in the research project entitled: "Acute effects of different forms of calcium on Bone resorption marker-CTX".
2. I have read the Participant Information Sheet, and the nature and purpose of the project and the risks inherent in my participation have been explained to me. I understand and agree to take part.
3. Although I understand that the purpose of this research project is to understand which form of calcium supplementation is better for improving bone health, it has also been explained that my involvement may not be of any direct benefit to me.
4. I have been given the opportunity to have a member of my family or a friend present while the project was explained to me.
5. I have been informed that, while information gained during the study may be published, I will not be identified and my personal results will not be divulged.
6. I understand that I am free to withdraw from the project at any time and that this will not affect my rights or the responsibilities of the researchers in any respect.
7. I understand that I will receive an honorarium of $200 as a thank you for my assistance in this research project only upon completion of the study.
8. I understand that all the information collected in this study will be stored on the university server in password protected files and in computers in buildings that are locked after hours. The information collected and generated during this study will be retained for 15 years as per Australian research retention guidelines.
9. I am aware that I should retain a copy of this Consent Form, when completed, and the attached Participant Information Sheet.
11. I provide consent for any abnormal results to be referred to my GP for follow-up. State Yes or NO

.....

(Signature) *(Date)*

WITNESS

I have described the nature and purpose of the research project and the risks inherent in participation to:

.... *(Name of subject).* In my opinion she/he understood the explanation.

Name... .....*(please print name)*

Status in Project: .....

.....

(Signature) *(Date)*

Appendix III Participant Information

UniSA | Research and Innovation Services

Participant Information

Name:

Contact number:

Emergency contact number:

Address:

Date of Birth:

Ethnicity:

Country of birth:

Medical History:

Current Medications:

Do you take calcium supplement: **YES** **NO**

If yes, how often:

Gynaecological History

- What was the approximate date of your last menstrual period?
- Have you received any medical treatment, such as a hysterectomy or chemotherapy that caused or precipitated menopause? **YES** **NO**
  - If yes, what treatment did you receive?
- Did you or do you currently take hormone replacement therapy (HRT)?
  - ( ) YES, I am currently on HRT
  - ( ) YES, I have taken HRT but do not currently
  - ( ) NO, I do not and have never taken HRT

Appendix IV Case Report Form

CCS 2017

Acute effects of supplemental and dietary calcium on Bone Resorption Marker (serum CTX, C-telopeptide of type 1 collagen) in lean and overweight post-menopausal women.

2017

CASE REPORT FORM

Volunteer ID: CCS XX

Investigators:
PhD candidate: Deepti Katyal Sharma
Supervisory team: Prof Howard Morris, Prof Peter Clifton and A/Prof Paul Anderson

UniSA ethics number: 36365
ANZCTR number: ACTRN12617000779370

PROTOCOL DEVIATIONS/VIOLATIONS

(Page 1 of 2)

Date (DD/MMM/YY)	Deviation/Violation	Explanation/Corrective measures/Comment	CRF Page #	Study personnel initials

CCS 2017

SCREENING VISIT

Visit 1 (Baseline)

1. Check Eligibility

a. Post-menopausal (at least 5 years). Record date/year of last menstrual cycle

b. Not on any osteoporotic medicine ☐

c. Taking calcium supplement Y ☐ N ☐

If yes, request to stop for a week before visit 2

d. Able to tolerate milk ☐

e. Non-diabetic ☐

2. Participant information sheet

Explain Participant information sheet ☐

3. Informed consent form sign off ☐

To be dated by volunteer and researcher

Date consent is obtained: ☐☐ ☐☐ ☐☐ (DD/MMM/YY)

Volunteer has been given a photocopy of the PICF Yes ☐ No ☐

4. Medications (active):

CCS 2017

ANTHROPOMETRIC DATA

	#1	#2	Average
Weight (kg)			
Height (cm)			

	#1	#2	Average
Waist Circumference (cm)			
Hip Circumference (cm)			

** Record two (2) measures of waist circumference. Where there is a discrepancy of 2% between the 2 measurements, a third measurement must be recorded and the average taken of the 3 measures.

Study personnel initials: ☐☐☐

Date ☐☐ / ☐☐ / ☐☐ Time ☐☐:☐☐

Randomization sequence:

Visit 2 (week 1)

BLOOD COLLECTION

Date & Time of last meal: **Fasting** **Yes** ☐ **No** ☐

How are you feeling today? _____

Have you taken any medication today? _____

Change of medications (since screening)? Yes ☐** No☐
Details :

Change of dietary supplements (since screening)? Yes ☐** No☐

Details :

Cannula insertion Arm Right ☐ Left ☐

Baseline Collect: 2 x 9 mL No Additive (white) Tube (ice) ☐
2 x 6 mL EDTA (purple) Tube (ice) ☐

Note: 2ml of blood to be collected/tube

Product and time of product administration:

Record time zero:

Collect Blood: 30 min, 1 hour, 2 hour, 3 hour, 4 hour, 5 hour

1 x No Additive (white) Tube
1 x EDTA (purple) Tube

5

CCS 2017

30 min	**Time:**
1 hour	**Time:**
2 hour	**Time:**
3 hour	**Time:**
4 hour	**Time:**
5 hour	**Time:**

Have all blood samples been obtained? Yes ☐ No ☐**

*** If No, please comment and record on page 2 any protocol deviations*

Blood Taking Code: Excellent ☐ Good ☐ Difficult ☐ Very Difficult ☐

Date ☐☐ / ☐☐ / ☐☐ (DD/MMM/YY) Collection Time ☐☐:☐☐ (HH:MM)

Visit 3 (week 2)

BLOOD COLLECTION

Date & Time of last meal: Fasting Yes ☐ No ☐

How are you feeling today? _____

Have you taken any medication today? _____

Change of medications (since screening)? Yes ☐** No ☐
Details :

Change of dietary supplements (since screening)? Yes ☐** No ☐

Details :

Cannula insertion Arm Right ☐ Left ☐

Baseline Collect: 2 x 9 mL No Additive (white) Tube (ice) ☐
2 x 6 mL EDTA (purple) Tube (ice) ☐

Product and time of product administration:

Record time zero:

Collect Blood: 30 min, 1 hour, 2 hour, 3 hour, 4 hour, 5 hour

1 x No Additive (white) Tube
1 x EDTA (purple) Tube

7

30 min	**Time:**
1 hour	**Time:**
2 hour	**Time:**
3 hour	**Time:**
4 hour	**Time:**
5 hour	**Time:**

Have all blood samples been obtained? Yes ☐ No ☐**

*** If No, please comment and record on page 2 any protocol deviations*

Blood Taking Code: Excellent ☐ Good ☐

Difficult ☐ Very Difficult ☐

Date ☐☐ / ☐☐ / ☐☐ (DD/MMM/YY) Collection Time ☐☐:☐☐ (HH:MM)

Visit 4 (week 3)

BLOOD COLLECTION

Date & Time of last meal: Fasting Yes ☐ No ☐

How are you feeling today? _____

Have you taken any medication today? _____

Change of medications (since screening)? Yes ☐** No ☐
Details :

Change of dietary supplements (since screening)? Yes ☐** No ☐

Details :

Cannula insertion Arm Right ☐ Left ☐

Baseline Collect: 2 x 9 mL No Additive (white) Tube (ice) ☐
2 x 6 mL EDTA (purple) Tube (ice) ☐

Product and time of product administration:

Record time zero:

Collect Blood: 30 min, 1 hour, 2 hour, 3 hour, 4 hour, 5 hour

1 x No Additive (white) Tube
1 x EDTA (purple) Tube

CCS 2017

30 min	**Time:**
1 hour	**Time:**
2 hour	**Time:**
3 hour	**Time:**
4 hour	**Time:**
5 hour	**Time:**

Have all blood samples been obtained? Yes ☐ No ☐**

*** If No, please comment and record on page 2 any protocol deviations*

Blood Taking Code: Excellent ☐ Good ☐ Difficult ☐ Very Difficult ☐

Date ☐☐ / ☐☐ / ☐☐ (DD/MMM/YY) Collection Time ☐☐:☐☐ (HH:MM)

DEXA

Since screening, has the volunteer had any radiation exposure for **research purposes**

(e.g. x-rays, radiotherapy, CT scan)? Yes ☐ No ☐

Date	Details

Check:

Bladder voided Yes ☐ No ☐

Jewellery removed (including glasses): Yes ☐ No ☐

Standard positioning: Yes ☐ No ☐

Wrapped: Yes ☐ No ☐

Scan Mode: ☐ Thin <13cm ☐ Standard 13-25cm ☐ Thick >25cm

Comments:

_____

_____

_____

Attach printouts on next page

Study personnel initials: ☐☐☐

Date ☐☐ / ☐☐ / ☐☐ (DD/MMM/YY) Time ☐☐:☐☐ (HH:MM)

Appendix V JCM Published Manuscript

Journal of
Clinical Medicine

MDPI

Article

Elevated Serum 25-Hydroxyvitamin D Levels Are Associated with Improved Bone Formation and Micro-Structural Measures in Elderly Hip Fracture Patients

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Abstract: Vitamin D, along with calcium, is generally considered necessary for bone health and reduction of fractures. However, he effects of improving vitamin D status have not always been observed to improve bone mineral density (BMD). We have investigated whether varying vitamin D status in humans, as measured by serum25(OH)D levels, relate to micro-structural and histomorphetric measures of bone quality and quantity, rather than density. Intertrochanteric trabecular bone biopsies and serum samples were collected from patients undergoing hip arthroplasty (65 females, 38 males, mean age 84.8 ± 8.3 years) at Royal Adelaide Hospital. Estimated GFR, serum ionized calcium, alkaline phosphatase, albumin, supplement and medication intake prior to surgery were taken from patient case records. Serum 25(OH)D, 1,25(OH)2D, and parathyroid hormone (PTH) levels were measured by immunoassays. Trabecular bone structural indices were determined by high-resolution micro-CT. Mean wall thickness (MWT) was measured on toluidine blue-stained histological sections. Bone mRNA levels for vitamin D metabolising enzymes CYP27B1 and CYP24A1 were measured by qRT-PCR. While serum 25(OH)D levels did not associate with bone volume/tissue volume

(BV/TV%), serum 25(OH)D levels were strongly and independently associated with MWT (r = 0.81 $p < 0.0001$)
with values significantly greater in patients with higher serum 25(OH)D levels. Furthermore, serum 25(OH)D levels were negatively associated with Bone Surface/Bone Volume (BS/BV) (r = −0.206, $p < 0.05$) and together with bone CYP27B1 and CYP24A1 mRNA accounted for 10% of the variability of BS/BV (p = 0.001). These data demonstrate that serum 25(OH)D is an independent positive predictor of micro-structural and bone formation measures and may be dependent, in part, on its metabolism
within the bone.

Keywords: 25-hydroxyvitamin D; bone surface/bone volume ratio; plate-like trabecular structure; mean wall thickness; CYP27B1; CYP24A1

1. Introduction

Vitamin D insufficiency and inadequate dietary calcium intake are prevalent worldwide and considered to be a public health issue. Inadequate vitamin D levels have been associated with poor muscle function, increased risk of falls, and osteoporotic fractures. Randomized controlled trials of vitamin D and calcium supplementation show divergent results depending on the clinical outcomes investigated. Studies which have evaluated bone mineral density as an end-point show minimal effect of supplementation [1] whereas studies which have investigated fracture as the clinical outcome show significant reductions in risk of fractures only when vitamin D and calcium are supplemented together but not when vitamin D is supplemented alone [2]. A statistically significant reduction of 30% in the risk of hip fracture and a 15% reduction in the risk of total fractures were reported in a recent meta-analysis of randomized controlled trials on calcium and vitamin D supplementation,
which included 30,970 participants [2]. Despite the available positive evidence, the significance of vitamin D in the prevention and treatment of osteoporosis with or without calcium supplementation remains debated, and its role in maintenance of bone health is yet to be fully explicated [3].

All current clinical evidence indicates that the beneficial effects of vitamin D on bone are related to the serum levels of the prohormone 25-dihydroxyvitamin D_3 (25(OH)D) and not 1,25-dihydroxyvitamin D_3 (1,25$(OH)_2$D), the active hormone, an apparent conundrum for the endocrine paradigm of vitamin D activity. In fact, the administration of 1,25$(OH)_2$D has been shown to be detrimental for bone health [4]. Direct effects of 25(OH)D in bone have been demonstrated with the three major bone cell types, i.e., osteoblasts, osteocytes and osteoclasts expressing genes for vitamin D metabolizing enzymes, 25-hydroxyvitamin D 1∝-hydroxylase (CYP27B1), the enzyme that catalyses the conversion of 25(OH)D to 1,25$(OH)_2$D and 25-hydroxyvitamin D 24-hydroxylase (CYP24A1), the enzyme that catalyses the degradation of 1,25$(OH)_2$D as well as vitamin D receptor (VDR) [5,6]. The demonstration that 25(OH)D is metabolized within osteoblasts and that this is required for mineralization, suggests that 25(OH)D could promote within bone mineralization in humans, independent of circulating 1,25$(OH)_2$D levels,

which provides a physiological context for this clinical observation, i.e., beneficial effect of prohormone 25(OH)D [5,6].
The aim of the present study was to investigate the relationships between serum 1,25$(OH)_2$D, 25(OH)D, parathyroid hormone (PTH), bone turnover markers on femoral bone structure in a cross-sectional study of elderly hip fracture patients. We also investigated the contribution of the bone CYP27B1 and CYP24A1 mRNA levels on the role of 25(OH)D in determining bone structure variables. We hypothesize that adequate vitamin D status results in local metabolism of 25(OH)D to 1,25$(OH)_2$D within bone, resulting in measures of improved bone health.

2. Experimental Section

2.1. Bone and Serum Specimens

Intertrochanteric trabecular bone biopsies, serum and presurgical radiographs were collected from 103 patients (65 females, 38 males, age 85.04 ± 8.4) undergoing hip arthroplasty at Royal Adelaide Hospital (Adelaide, South Australia) between August 2015 and January 2017 due to experiencing a fracture of the neck of femur (NOF). During preoperative preparation, patients or next of kin were asked to participate in this study, and written consent was obtained. Date of surgery, patient age,
weight, height, comorbidities, eGFR (MRD-EPI equation), medication history, total plasma levels of ionized calcium (estimated) [7], albumin and total alkaline phosphatase were collected from patient case reports. Patients that were being treated with anti-resorptive or calcitriol therapy were excluded from the study. The ethics approval for this study was obtained from Royal Adelaide Hospital Ethics Committee (Protocol no 130114; HREC/13/RAH/33), and institutional ethics approval was obtained from University of South Australia human ethics committee (HREC 35360).

2.2. Serum Analyses

Single blood specimen was collected at the time of surgery in SST tubes (serum separator tubes), centrifuged at 5000 rpm for 10 min to collect serum and stored at −80 °C until required. Serum samples were analyzed by chemiluminescent immunoassay (Diasorin Liaison) for 25(OH)D, 1,25$(OH)_2$D,
intact PTH, osteocalcin and bone-specific alkaline phosphatase.

2.3. Bone Biopsy Preparation

The trabecular bone biopsies were cut into two halves with one half fixed in formalin (10% formalin) for 7 days at 4 °C followed by storage in 70% ethanol at room temperature until microstructural analyses were conducted. The other half was submerged in RNA later and stored at −80 °C for gene expression analyses.

2.4. Bone Biopsy Micro-Computed Tomography

Trabecular bone specimens were assessed for bone mass and bone quality variables with the 1076 Bruker micro-CT imaging system. The X-ray tube was operated at 60 KV and 167 µA with a resolution of 18 µm with a 0.5 mm aluminium filter and exposure time of 1770 ms. The images were reconstructed using the CTAn software (v1.13, Kontich, Belgium) after correcting for smoothing (1), beam hardening (30%) and ring artifact reduction (20). The specimens varied considerable in volume (1093.7 ± 511.2 mm^3; range: 291.7–1078.8 mm^3); hence, using a uniformly sized region of interest could have potentially oversampled the shorter bone and undersampled the longer bone. Hence, a slice-by-slice analysis tracing approach was adopted to maximize the volume available for analysis which included all trabecular bone and excluded

cortical bone and marrow space. Morphometric indices were determined using an adaptive thresholding algorithm with a pre-thresholding of 60 to 255 and a radius of 8. Variables such as bone volume/tissue volume (BV/TV%), trabecular number (Tb.N), and trabecular separation (Tb.Sp) describe trabecular bone quantity or mass. BV/TV% and Tb.N are measures of the amount of trabecular bone within a selected volume of cancellous tissue. However, in addition to the quantity of bone, the trabecular bone quality plays a critical role in determining bone strength and is determined by the three-dimensional orientation and connectivity of individual trabeculae. Trabecular bone quality measures include bone surface/bone volume ratio (BS/BV) and trabecular thickness (Tb.Th). The measurement of BS/BV provides an integrated measure of thickness and texture of the bone.

2.5. Bone Biopsy Histology

Trabecular bone biopsies were then processed through 70% to 100% ethanol to dehydrate the sample and then placed in 5 mL solution containing 100% methylmethacrylate (MMA) and 10% *v*/*v* polyethylene glycol (PEG) and stored at room temperature for 10–14 days. Resin embedding then occurred by a solution of 100% MMA, 10% PEG and 0.4% peroxydicarbonate. Biopsies were submerged in 5 mL of this solution and placed in at 37 °C for 24 h to allow the resin to harden. A volume of 5 µm thick decalcified sections were cut and stained by Haematoxylin and Eosin [8]. Mean wall thickness, a measure of completed packets of bone remodeling, were measured in each section (Figure A1) (adapted from [9]). Wall thickness was defined as a complete trabecular bone packet on straight, rod-like trabecular structures. Five sites (10 measurements per site) per section were obtained between perpendicular cement lines beneath bone where no resorption of osteoid formation occurred.

2.6. Radiographic Cortical Bone Analyses

Cortical bone measurements: Anterior-Posterior plain radiographs of the contralateral uninjured limb were used to measure average medial (M) and lateral (L) cortical thicknesses and M-L medullary width and femoral width using CARESTREAM software (Figure A2).

2.7. Bone mRNA Analyses

Total RNA was extracted from the pulverized bone using the TRIZOL reagent (Life-technologies, Carlsbad, CA, USA), and cDNA samples were synthesized (Superscript-III kit, Life Technologies). The mRNA expression of genes of interest was measured by real-time PCR using iQ SYBR Green Supermix (Bio-Rad Laboratories, Hercules, CA, USA), as per the manufacturer's instructions. Relative gene expression was quantified delta-CT method and normalizing to the expression of β-actin mRNA. The primer sequence for CYP27B1 is TGGCCCAGATCCTAACACATTT (forward) GTCCGGGTCTTGGGTCTAACT (reverse) CYP24A1 CCTGCTGCCAGATTCTCTGGAA (forward) TTGCCATACTTCTTGTGGTACTCC (reverse) and Beta actin CTGCCCTGAGGCACTCTT (forward) AGTTTCGTGGATGCCACAGG (reverse).

2.8. Statistical Analyses

Statistical analysis was performed using the SPSS statistical Package (IBM SPSS Stats 25, St. Leonards, Australia). Some of the variables were not normally distributed, hence values are expressed as median and interquartile range. Spearman's correlation coefficient was calculated to determine correlations between structural, metabolic and gene expression variables. The metabolic variables which correlated with structural variables were tested through step-wise multiple linear regression analyses to identify metabolic determinants of bone mass and bone quality variables. Independent samples Kruskal–Wallis test was used to compare bone structural and quality variables between various 25(OH)D intervals. The significance level was set at 0.05.

3. Results

3.1. Biochemical Analyses

The median serum 25(OH)D levels were 52.7nmol/ L (Table 1). Serum 25(OH)D levels correlated positively with circulating 1,25(OH)$_2$D (r = +0.311, p < 0.05) suggesting substrate insufficiency in a significant proportion of the patients. Serum 25(OH)D levels were not associated with eGFR or other measured biochemical parameters. However, serum 1,25(OH)$_2$D levels were determined by a combination of serum 25(OH)D, PTH levels and eGFR (Multiple R^2 = 0.235, p < 0.01) (Table 2).

Negative correlations were observed between eGFR and age (r = −0.464, p < 0.001) and eGFR and PTH

(r = −0.387, p < 0.001), consistent with a decline in renal function with age resulting in higher PTH values, which contributed to increased serum 1,25(OH)$_2$D levels. Serum osteocalcin, a marker of bone turnover, correlated negatively with eGFR (r = −0.369, p < 0.001) suggesting that compromised kidney functions in these patients is associated with higher bone turnover.

Table 1. Biochemical profile of the participants.

	Median (IQ Range)	Range
Age, years	86.5 (80.3–90.3)	63.0–102.7
eGFR, mL/min/1.73 m^2	70 (47–84)	11.0–90+
Ionized Ca, mmol/L	1.19 (1.15–1.23)	1.03–1.37
25(OH)D, nmol/L	52.7 (30.4–73.4)	10–225
1,25(OH)2D, pmol/L	93.8 (67.1–129.8)	18.4–229
PTH, pmol/L	4.9 (3.28–9.43)	1.1–32.7
Osteocalcin, nmol/L	2.41 (1.21–3.24)	0.33–21.8
BS-ALP, µg/L	13.3 (9.78–17.23)	3.45–36.30

PTH: parathyroid hormone.

Table 2. Determinants of serum 1,25(OH)$_2$D- Multiple linear regression analyses.

Dependent Variable	Independent Variables	Standardized Coefficients (beta)	p-Value
Serum 1,25(OH)$_2$D	25(OH)D	0.332	<0.001
	+PTH	0.272	0.006
	+eGFR	0.439	<0.001
	+Constant	+0.187	0.992
		Combined R^2 = 0.235	<0.001

3.2. Relationship between Serum Vitamin D Levels and Bone Structure and Histomorphometric Measures

Serum 25(OH)D levels did not correlate with the bone structure variables of BV/TV%, Tb.Th and Tb.N by either linear analyses nor when serum 25(OH)D levels were grouped by intervals of 25(OH)D adequacy ranging from deplete to replete (Figure 1A–C). However, a trend for serum 25(OH)D levels association with Tb.Th was observed (r = 0.19, p = 0.06). Neither serum 1,25(OH)$_2$D or PTH correlated with BV/TV%, Tb.N or Tb.Th. Mean wall thickness (MWT), a dynamic marker of completed bone formation, increased consistently and significantly with increasing vitamin D levels (Figure 1D, Table A1). MWT trended to be higher in patients with 25(OH)D greater than 70 nmol/L as compared to patients with serum 25(OH)D between 30–50 nmol/L (p = 0.021, adjusted p = 0.12) (Table A1). Furthermore, MWT exhibited a positive linear

correlation with 25(OH)D levels (r = 0.810, p < 0.0001) (Figure A3). Although, MWT was also correlated with serum 1,25(OH)$_2$D levels, only serum 25(OH)D determined MWT values in a multiple linear regression model (Table 3). Bone surface/bone volume (BS/BV) ratio, a measure of bone texture and a surrogate marker of plate-like trabecular structure, was negatively associated with serum 25(OH)D (r = −0.206, p < 0.05), indicative of higher vitamin D levels resulting in more plate-like structures. However, the BS/BV values did not reach statistical significance when divided by serum 25(OH)D intervals (Figure 1E). Neither serum 1,25(OH)$_2$D nor PTH correlated with BS/BV (p = 0.9 and 0.8, respectively). With respect to cortical bone measurements, serum 25(OH)D levels correlated negatively with total femoral diameter (r = −0.239, p = 0.018) and trend only for medullary width (r = −0.169, p = 0.09). None of the cortical parameters differ significantly across vitamin D intervals (Figure 1F,H). Neither serum 1,25(OH)$_2$D nor PTH contributed to the variation in any cortical measure.

Table 3. Multiple linear regression analyses for biochemical determinants of Mean Wall Thickness.

Dependent Variable	Independent Variables	Standardized Coefficients (beta)	*p*-Value
MWT	25(OH)D	0.745	<0.001
	+1,25(OH)$_2$D	+0.095	0.190 (NS)
	+5.4		
		Adjusted R^2 = 0.602	p < 0.001

MWT: Mean wall thickness.

3.3. Contribution of CYP27B1 and CYP24A1 mRNA Expression in Bone to Histomorphometric Measures

To determine whether bone-specific expression of vitamin D metabolizing enzymes, CYP27B1 and CYP24A1 contribute to the relationship of serum 25(OH)D with either MWT or BS/BV, multiple linear regression analyses were carried out to investigate the variance accounted by these measures. Bone CYP27B1 and CYP24A1 mRNA levels did not contribute to the serum 25(OH)D determination of MWT (Table 4). However, bone CYP24A1 mRNA contributed positively (p = 0.02) and CYP27B1 mRNA negatively; as a trend, only (p = 0.1) contributed to the serum 25(OH)D determination of BS/BV (Table 5).

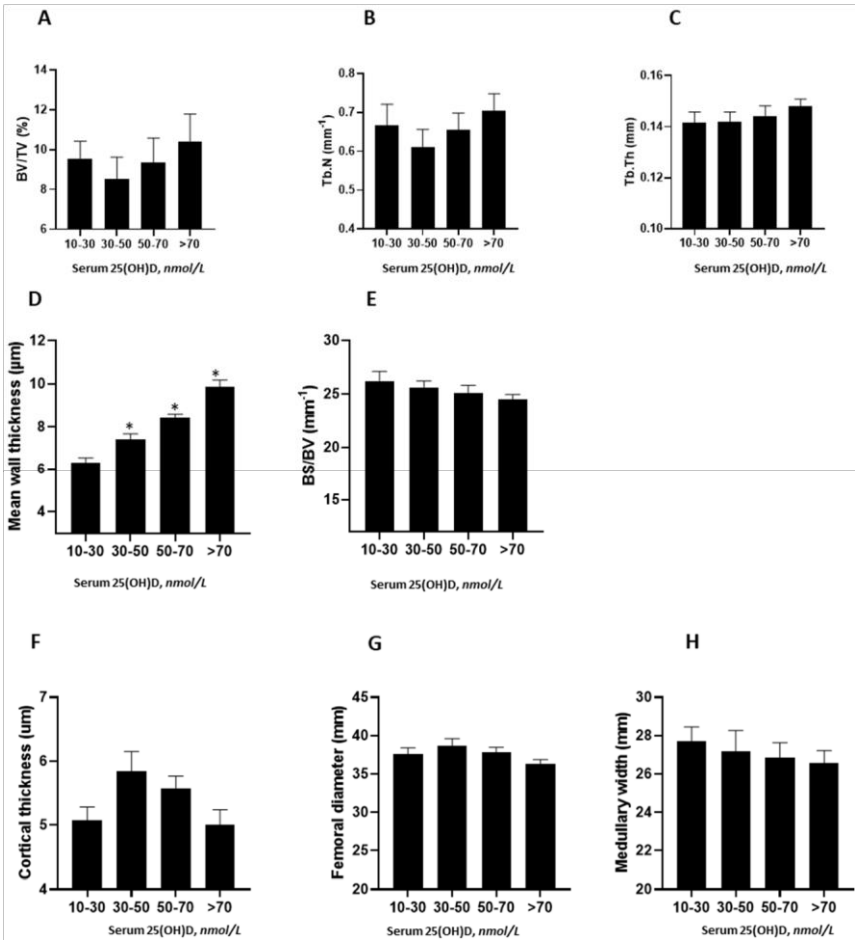

Figure 1. Trabecular and cortical bone structural variables across 25-dihydroxyvitamin D3 (25(OH)D intervals with (**A**) BV/TV (bone volume/tissue volume), (**B**) Tb.N (trabecular number) and (**C**) Tb.th (trabecular thickness), (**D**) MWT (mean wall thickness), (**E**) BS/BV (bone surface/bone volume), (**F**) cortical thickness, (**G**) femoral diameter and (**H**) medullary width. Mean +/− SEM; n = 103; * $p < 0.05$ vs. 10–30 nmol/L serum 25(OH)D interval.

Table 4. Multiple linear regression analyses for serum 25(OH)D, CYP27B1 and CYP24A1mRNA levels as determinants of Mean Wall Thickness.

Dependent Variable	Independent Variables	Standardized Coefficients (beta)	*p*-Value
MWT	25(OH)D	0.743	<0.001
	+CYP27B1	+0.043	0.655 (NS)
	+CYP24A1	+0.064	0.504 (NS)
	+5.1		
		Adjusted R^2 = 0.54	$p < 0.001$

Table 5. Multiple linear regression analyses for serum 25(OH)D, CYP27B1 and CYP24A1mRNA levels as determinants of Bone Surface/Bone Volume.

Dependent Variable	Independent Variables	Standardized Coefficients (beta)	*p*-Value
BS/BV	25(OH)D	−0.294	0.012
	+CYP27B1	−0.208	0.115
	+CYP24A1	+0.306	0.021

	+27.5		
		Adjusted R^2 = 0.104	0.016

BS/BV: Bone surface/bone volume.

4. Discussion

Vitamin D deficiency, as measured by serum 25(OH)D levels, has been shown to be associated with increased risk of hip fracture in the elderly [10]. Despite this knowledge, questions remain as to mechanisms by which higher 25(OH)D levels improve skeletal health [11], especially since levels above 20 nmol/L are considered adequate for renal $1,25(OH)_2D$ synthesis and for intestinal calcium absorption [12]. In clinical studies, the effects of vitamin D status and nutritional supplementation in skeletal health have been largely limited to measures of BMD and fracture incidence. However, the imprecision of BMD as a measure of fracture risk [13] and the short-term follow-up period of supplementation trials are among the potential limiting factors when determining the effects of vitamin D on skeletal health.

An incomplete understanding of the mechanistic actions of how vitamin D, in particular 25(OH)D, mediates positive effects on the skeleton also contributes to the uncertainty for the need to recommend levels of serum 25(OH)D, level above which is required for renal $1,25(OH)_2D$ synthesis. The association between eGFR and circulating $1,25(OH)_2D$ levels in this elderly cohort is consistent with the role renal function has in determining vitamin D status [14]. Interestingly, while serum $1,25(OH)_2D$ levels are determined by eGFR, in addition to PTH and 25(OH)D levels, no bone parameters were determined by serum $1,25(OH)_2D$ levels, suggesting that changes to 25(OH)D levels were more important to determine MWT and BS/BV.

The strong positive relationship between serum 25(OH)D levels and MWT suggests that osteoblastic bone formation was improved in the presence of higher serum 25(OH)D levels. Serum 25(OH)D levels above 50 nmol/L significantly increase MWT. While it is possible that serum 25(OH)D levels of 70 nmol/L and above also increase MWT group (p = 0.021), when we adjusted for multiple comparisons, the significance was (p = 0.2), perhaps due to smaller number of participants per group. BS/BV, an indicator of texture and strength of bone, was negatively associated with 25(OH)D levels suggesting that patients with adequate vitamin D status had plate-like structure consistent with higher bone strength. Significantly lower BS/BV and Tb.Pf values were reported in a recent clinical study examining the effect of bisphosphonate treatment on femoral head trabecular bone micro-architecture in non-treated fracture controls as compared to hip-fracture patients. Authors concluded that lower values for both the variables suggest preserved connectivity of trabecular bone in the bisphosphonate group. More importantly, Tb.Pf and BS/BV were strongly correlated with measures of bone strength [15]. A lower BS/BV may also signify decrease bone resorption [15]; hence, a lower BS/BV with higher vitamin D levels observed in our study is consistent with structural effects of vitamin D on reducing bone resorption.

We have previously proposed, using animal and cell culture studies, that the direct supply of 25(OH)D to bone is required for local $1,25(OH)_2D$ synthesis [5,16,17] and contributes to the regulation of bone formation [18] and resorption [19,20] processes. Few studies to date have investigated the relationship between 25(OH)D status and local metabolism of vitamin D within human bone. Elderly women with subcapital femur fracture have been shown to exhibit five-times lower levels of bone-derived $1,25(OH)_2D$ levels when compared with aged-matched women without fracture, despite comparable circulating levels of $1,25(OH)_2D$ [21]. We have previously shown that using eleven human biopsies, the relationship of CYP27B1, CYP24A1 and VDR mRNA levels with genes, including vitamin D responsive genes involved in bone remodeling [22]. Furthermore, we have shown that human primary osteoblasts synthesize $1,25(OH)_2D$ from 25(OH)D via CYP27B1 activity, which directly promotes mineralization [23]. While these studies are indicative of the requirement for 25(OH)D to promote bone health, the question of changes

in human structural bone in relation to serum 25(OH)D is less clear. In the present study, we have demonstrated that adequate serum 25(OH)D levels along with bone mRNA levels, which are higher for CYP27B1 and lower for CYP24A1, is associated with reduced BS/BV, which is indicative of a stronger trabecular bone structure. Our findings concur with a recent clinical study in a Danish cohort of post-menopausal women that showed daily supplementation with vitamin D3 (2800IU) resulted in significantly improved bone strength but did not affect the areal BMD [24].

5. Conclusions

In conclusion, we have demonstrated that higher serum 25(OH)D levels are associated with improved bone formation. The association of serum 25(OH)D levels with bone quality variables of MWT and BS/BV, rather than bone mass variables, suggests that determining the efficacy of vitamin D supplementation on bone health should take in consideration measures of bone quality beyond bone mineral density. Furthermore, higher 25(OH)D along with higher expression of bone CYP27B1 and lower expression of CYP24A1 is associated with stronger plate-like structure and supports the paradigm of bone supply of $1,25(OH)_2D$ levels to improve bone health.

Author Contributions: Conceptualization, D.K.S., P.M.C., H.A.M. and P.H.A.; methodology, D.K.S., R.K.S., R.S., P.M.C., H.M.A. and P.H.A.; formal analysis, D.K.S., R.K.S., R.S., P.M.C., H.A.M. and P.H.A.; resources, T.S.R., L.B.S., G.J.A., H.A.M. and P.H.A.; data curation, D.K.S., P.M.C., H.A.M. and P.H.A.; writing—original draft preparation, D.K.S. and P.H.A.; writing—review and editing, D.K.S., T.S.R., R.S., P.C.M. and P.H.A.; visualization, D.K.S., R.K.S., P.M.C. and P.H.A.; supervision, L.B.S., G.J.A., P.H.C., H.A.M. and P.H.A.; project administration, H.A.M. and P.H.A.; funding acquisition, P.M.C., H.A.M. and P.H.A.

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Appendix A

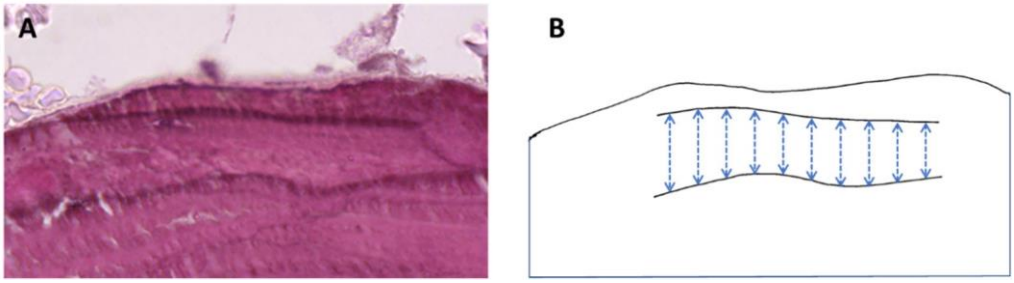

Figure A1. (**A**) Representative histological section indicating packet of trabecular bone and (**B**) representative measurements between cement lines.

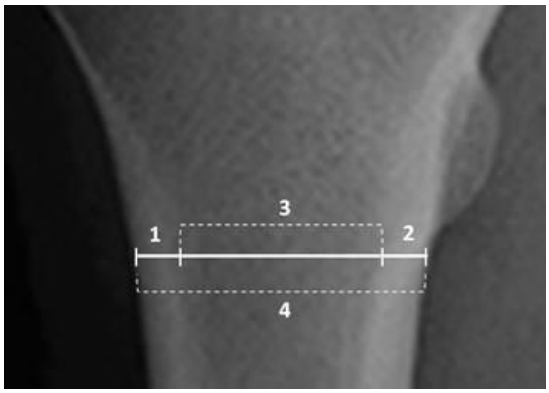

Figure A2. Representative femoral Anterior–Posterior plain radiograph indicating the measures of lateral cortical thickness (1), medial cortical thickness (2), M-L medullary width (3) and femoral width (4).

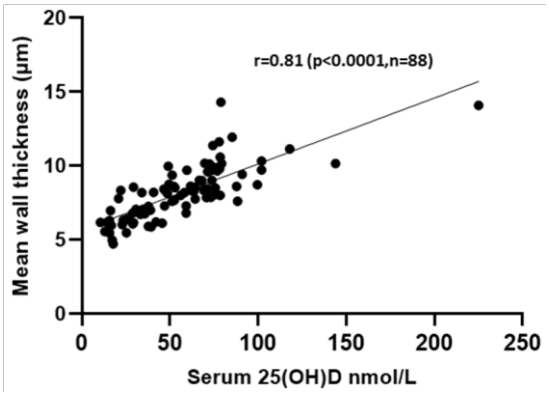

Figure A3. Scatter plot between serum 25(OH)D and mean wall thickness.

Table A1. Comparison of Mean Wall Thickness across 25(OH)D intervals (Kruskal-Wallis test).

25(OH)D Interval nmol/L (Number of Patients)	Median 25(OH)D Levels (nmol/L)	Test Statistics	*p* Value (vs.>70 nmol/L)	Adjusted *p* Value [a] (vs. >70 nmol/L)
10–30 (21)	6.17	−50.31	<0.001	<0.001
30–50 (18)	7.16	−34.77	<0.001	<0.001
50–70 (20)	8.40	−17.17	0.021	0.12
>70 (29)	9.70			

[a] Significance values have been adjusted by the Bonferroni correction for multiple tests.

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