UNIVERSITY OF TECHNOLOGY SYDNEY

Genetic and Environmental Determinants of Bone Phenotypes

By Ngoc Bao Huynh

A thesis submitted to the University of Technology Sydney in partial fulfilment of the requirements for the degree of

Doctor of Philosophy

Under the supervision of Distinguished Professor Tuan Van Nguyen and Associate Professor Nham Tran

School of Biomedical Engineering

Faculty of Engineering and Information Technology

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Certificate of Original Authorship

I, Ngoc Huynh, declare that this thesis is submitted in fulfilment of the requirements for the award of Doctor of Philosophy, in the School of Biomedical Engineering, Faculty of Engineering and IT, at the University of Technology Sydney.

This thesis is wholly my own work unless otherwise referenced or acknowledged. In addition, I certify that all information sources and literature used are indicated in the thesis.

This document has not been submitted for qualifications at any other academic institution.

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Impact of and Response to the COVID-19 Pandemic on this Thesis

In May 2021, I received an offer to pursue my PhD studies at the University of Technology Sydney (UTS). The initial plan was to relocate to Sydney, Australia, to attend coursework and conduct laboratory research at UTS, with an expected completion time of three years. However, the COVID-19 pandemic significantly disrupted this timeline, causing a six-month delay and preventing my travel to UTS and access to laboratory facilities.

Faced with these obstacles, I adapted my approach. I used this unexpected period to deepen my understanding of osteoporosis research and enhance my data analysis skills. This pivot proved fruitful, as I successfully completed a data analysis for my first paper on the Collagen type 1 alpha 1 gene (COLIA1), a crucial gene in osteoporosis studies. This work not only formed the basis for Chapter 4 of my thesis but also allowed me to identify potential research aims. By the end of this challenging period, I had successfully presented my findings at a conference, demonstrating my ability to progress despite unforeseen circumstances.

Publication

Huynh, N., De Dios, K., Tran, T.S. *et al.* Association between the Sp1-binding-site polymorphism in the collagen type I alpha 1 (*COLIA1*) gene and bone phenotypes: the Dubbo Osteoporosis Epidemiology Study. *J Bone Miner Metab* (2024). https://doi.org/10.1007/s00774-024-01558-8

Publications and Awards Arising from this Study

The following publications and awards arising from the works conducted during the candidature.

Published papers

1. **Huynh, N**., De Dios, K., Tran, T.S., Center, J.R., & Nguyen, T.V. Association between the Sp1-binding-site polymorphism in the collagen type I alpha 1 (*COLIA1*) gene and bone phenotypes: the Dubbo Osteoporosis Epidemiology Study. *J Bone Miner Metab* (2024). https://doi.org/10.1007/s00774-024-01558-8
2. Dios, K., **Huynh, N**., Tran, T. S., Center, J. R. & Nguyen, T. V. Association between Fat Mass and Obesity-Related Transcript Polymorphisms and Osteoporosis Phenotypes. *J Bone Metab* 31, 48-55 (2024). https://doi.org:10.11005/jbm.2024.31.1.48

Manuscripts submitted or in preparation

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Oral presentations

1. Novel Genetic Variants Associated with Bone Mineral Density. Endocrine Society of Australia (ESA), Society for Reproductive Biology (SRB), Australian and New Zealand Bone and Mineral Society (ANZBMS) Joint Scientific Meeting in Adelaide, Australia: 10 – 13 November 2024.

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Poster presentations

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2. Estimating “Skeletal Age” by Bone Loss in Elderly Men and Women. Annual Scientific Meeting of Australian and New Zealand Bone and Mineral Society in Newcastle, Australia: 22 – 25 October 2023.

3. Estimating 'Skeletal Age' by Bone Loss in Elderly Men and Women. Annual Meeting of American Society for Bone and Mineral Research in Vancouver, Canada: 13 – 16 October 2023.

4. Bone Loss in Elderly Women is Related to Lifestyle Factors. Annual Meeting of American Society for Bone and Mineral Research in Austin, Texas: 9 – 12 September 2022.

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List of Abbreviations

ANOVA	Analysis of variance
ATXN10	Ataxin 10
BMD	Bone mineral density
CCDC170	Coiled-coil domain containing 170
CI	Confidence interval
COLIA1	Collagen type I α1 gene
CV	Coefficient of variation
DNA	Deoxyribonucleic acid
DOB	Date of birth
DOES	Dubbo Osteoporosis Epidemiology Study
DXA	Dual X-ray absorptiometry
DZ	Dizygotic twins
FAM3C	Family with sequence similarity 3, member C
FFQ	Block Food Frequency Questionnaire
FNBMD	Femoral neck bone mineral density
GCTA	Genome-wide Complex Trait Analysis
GPRASP1	G protein-coupled receptor associated sorting protein 1
GWAS	Genome-wide association study
HMGA1P6	High mobility group AT-hook 1 pseudogene 6
HTOTBMD	Total hip bone mineral density
HWE	Hardy-Weinberg equilibrium
HZ	Hazard ratio
IGFBP-3	Insulin-like growth factor-binding protein-3
LINC01234	Long intergenic non-protein coding RNA 1234
LINC02131	Long intergenic non-protein coding RNA 2131
LSBMD	Lumbar spine bone mineral density
MRFPP1	Mitochondrial ribosome recycling factor pseudogene 1
MROS	Osteoporotic Fractures in Men Study

MZ	Monozygotic twins
PASE	Physical Activity Scale for the Elderly
PCR	Polymerase chain reaction
PRS	Polygenic risk scores
PRS-CS	Polygenic Risk Score Continuous Shrinkage
PTH	Parathyroid hormone
SD	Standard deviation
SNP	Single nucleotide polymorphism
SORCS2	Sortilin Related VPS10 Domain Containing Receptor 2
SOF	Study of Osteoporotic Fractures
VOS	Vietnam Osteoporosis Study
VPS10	Vacuolar protein sorting 10
WNT16	Wnt family member 16

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Abstract

Osteoporosis is a metabolic disease characterized by reduced bone strength and deteriorating bone structure, leading to an increased risk of fragility fractures, which in turn, signal an increased risk of refracture and mortality. The lifetime fracture risk of fracture is approximately 1 in 2 for women and 1 in 3 for men, with around 9 million osteoporosis-related fractures occurring globally each year, making osteoporosis a serious public health concern. Bone mineral density (BMD) is the most robust risk factor for fractures, yet BMD loss in the elderly varies widely among individuals. Although twin studies indicate that genetic factors influence this variation, the extent of their impact remains poorly understood. This study aimed to investigate the contributions of specific genetic variants and lifestyle factors to BMD, bone loss, and fracture risk, to improve early detection, prevention, and treatment strategies for osteoporosis. The present study was based on data from four major cohorts: the Dubbo Osteoporosis Epidemiology Study (DOES), the Study of Osteoporotic Fractures (SOF), the Osteoporotic Fractures in Men Study (MrOS), and the Vietnam Osteoporosis Study (VOS). The cohorts included over 10,000 participants aged 60 years and older with repeated BMD measurements. Study 1 tested the hypothesis that increased bone loss is a risk factor for mortality, finding that rapid declines in femoral neck BMD significantly increased mortality risk, while stable or slightly improved BMD reduced it, highlighting BMD maintenance as a marker for healthy aging. Study 2 examined lifestyle factors, showing that smoking accelerated bone loss, while alcohol consumption, regular physical activity, and adequate dietary calcium intake reduced it. Study 3 examined the association between bone loss and COLIA1 gene polymorphisms, finding the Sp1 TT genotype doubled fracture risk but was not linked to bone loss, suggesting a role in bone fragility independent of bone loss. Study 4 was a genome-wide association analysis that identified 16 genetic variants associated with BMD in individuals of Southeast Asian descent, including novel variants in genes such as SORCS2, LINC02131, and ATXN10, accounting for 0.6% to 1.3% of BMD heritability. Collectively, these studies underscore the multifactorial nature of bone loss and fracture risk, suggesting that combining genetic data with clinical and lifestyle factors could enhance fracture risk prediction and support personalized osteoporosis management strategies.

CHAPTER 1

INTRODUCTION

1 Introduction

1.1 Osteoporosis and Osteoporotic Fracture

1.1.1 Definition of osteoporosis

Osteoporosis is one of the most common musculoskeletal disorders in the elderly population globally. Conceptually, the disease is characterized by low bone mass and deterioration of bone microstructure, weakening bone tension and strength and an increased risk of fracture. Operationally, this definition is translated into bone mineral density (BMD) T-score by the World Health Organization, which will be reviewed below (section 1.3.2.4).

1.1.2 Economic cost of osteoporosis

Fractures resulting from osteoporosis pose a significant burden on individuals, affecting their quality of life, as well as on the public health system due to the associated economic costs. In Italy, for example, the National Health Service and Social Security System reported medical expenses for osteoporosis treatment reaching approximately €2.2 billion in 2017, averaging around €8691 per patient annually [1]. In the United States, more than $16.9 billion was spent on treating over 2.0 million fracture cases in 2005 [2].

In the Netherlands, healthcare expenses related to hip fractures costed €102 million for men and €276 million for women in 2007 [3]. Over a decade, these costs increased by 17%, reaching €130 million and €331 million respectively by 2017 [4]. Osteoporosis also represents a growing economic concern in China, where costs related to 2.33 million osteoporotic fractures in 2010 costed $9.45 billion, with projections indicating that costs could rise to $25.43 billion by 2050 for an estimated 5.99 million fractures [5].

In Australia, the direct expenditure for osteoporosis-linked fractures in the population aged 50 and above was $1.76 billion in 2012 [6]. A separate study by Tatangelo et al. (2019) reported that in 2017, the direct cost of different fracture sites in the Australian population

aged 50 and older was AUD $3.44 billion. Hip fractures alone accounted for $1.01 billion, with wrist fractures at $164 million, vertebral fractures at $267 million, and fractures at other sites at $903 million [7]. This study also detailed the costs of osteoporosis, osteopenia, and associated fractures across various Australian states (Figure 1.1).

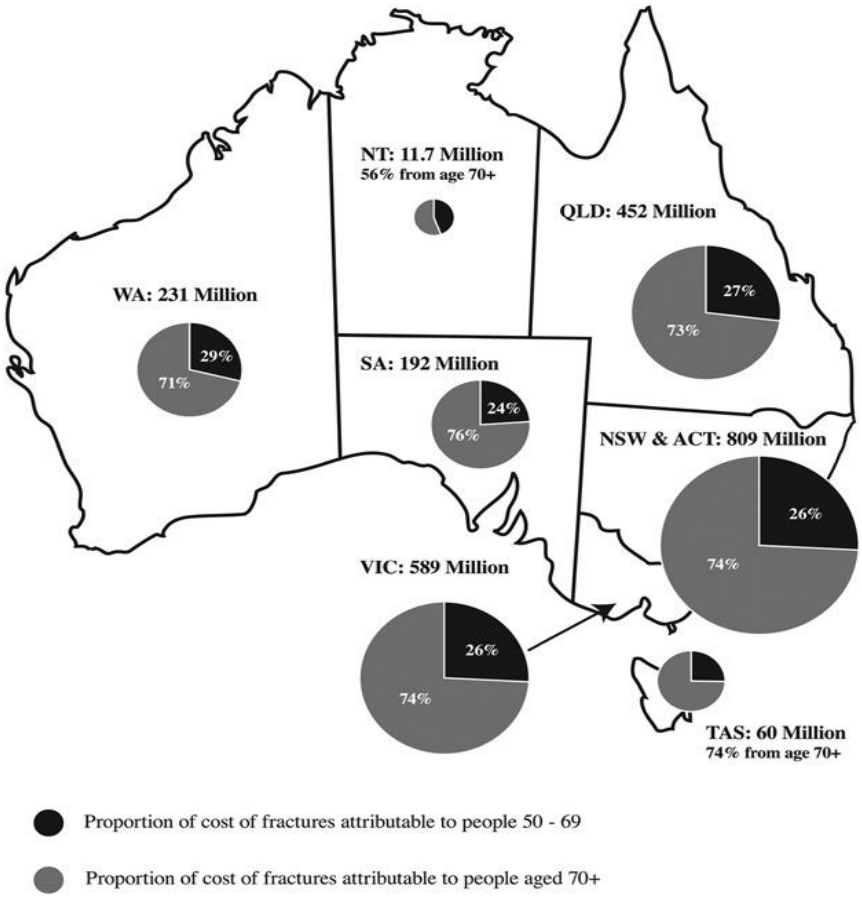

Figure 1.1 Osteoporosis, osteopenia, and fracture expenses across different states in 2017. Figure reprinted from Tatangelo et al., 2019

The economic burden of fractures continues to rise as fracture incidence increases year by year (Figure 1.2) [8]. The European Commission forecasts a 135% increase in hip fracture incidence and a 57% increase in vertebral fractures over the next 50 years, expecting the costs for fractures treatment to rise by about €76.8 billion in 2050 [9, 10]. In Switzerland, there were about 74,000 reported fracture cases in 2001, resulting in a total cost of CHF 2,050 million for treatment, care, and prevention. It is projected that these costs will rise by 29% to CHF 2,642 million by 2025, with an estimated 98,786 fracture cases [11]. A study by Tatangelo et al. in Australia reported that the direct cost of osteoporosis-related fractures consumed AUD 3.44 billion (USD 2.77 billion) in 2017, which was nearly twice the expenditure in 2007, about AUD 1.9 billion [12].

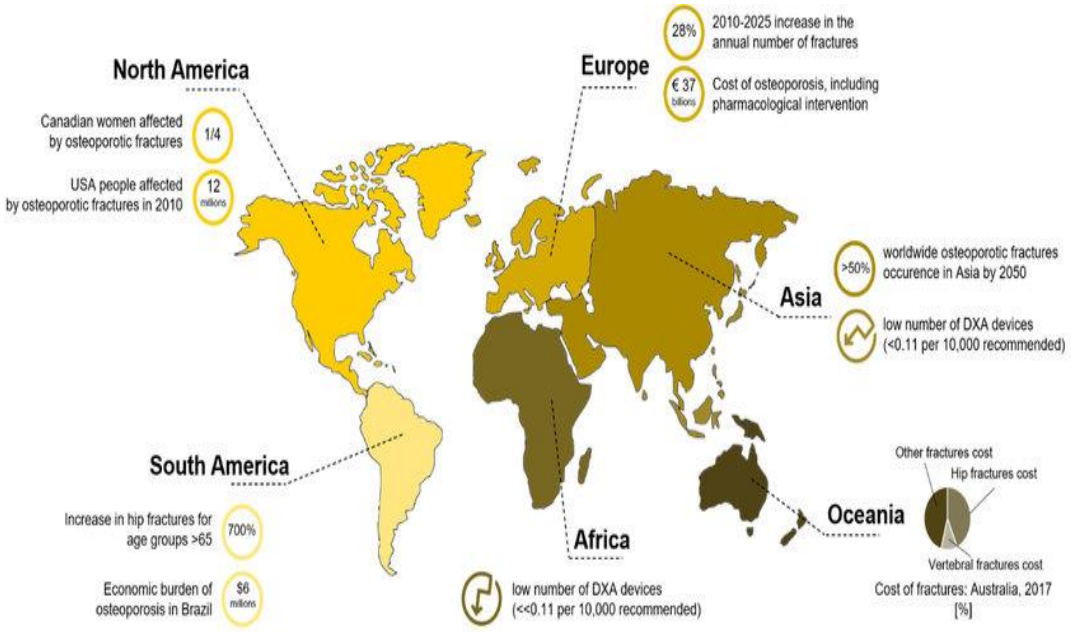

Figure 1.2 Visual representation illustrating the global impact of osteoporotic fractures. Figure reprinted from Buccino et al., 2021

1.1.2.1 Impact of aging on economic burden

Age is a critical factor in determining the economic burden of osteoporotic fractures. As life expectancy rises, demographic patterns are shifting, with a substantial increase in the older population. The global population of individuals aged 65 and older is expected to rise from 323 million in 1990 to over 1.5 billion by 2025 [13]. This demographic shift presents significant challenges for healthcare systems worldwide.

As people age, the incidence of fractures rises due to a natural decline in bone mineral density (BMD) and increased susceptibility to falls. According to a study by Cooper et al., the number of hip fractures among people aged 35 and older is projected to increase dramatically, from 1.66 million cases in 1990 to approximately 6.26 million by 2050 [14]. In the United States alone, the population of individuals aged 65 and older is expected to grow from 32 million in 1990 to 69 million by 2050. Assuming age-specific fracture risks remain constant, this increase in the elderly population could lead to a nearly threefold rise in hip fracture cases [14]. Hip fractures are among the most expensive osteoporotic injuries to treat, often requiring surgical intervention, long-term care, and rehabilitation, thereby contributing significantly to the overall economic burden of osteoporosis [15].

1.1.2.2 Influence of competing risks on costs

However, estimates of the long-term economic burden of osteoporosis may be inflated if competing risks are not considered. While fracture risk is often assessed over extended periods (5 or 10 years), the competing risk of mortality—particularly among elderly individuals—can lead to an overestimation of future fracture rates and their associated costs. A study by Bliuc et al. (2013) demonstrated the significant cumulative risk of mortality following low-trauma fractures, with 51% of men and 39% of women dying within 5 years post-fracture [16]. A substantial portion of this mortality (27% in men and 24% in women) exceeded the expected rates for an age- and sex-matched population [16]. Failing to account for the competing risk of death when modeling the long-term economic impact of osteoporosis may inflate the projected costs, as it overlooks the likelihood that many individuals may not live long enough to experience refractures or incur prolonged care costs.

1.1.2.3 Personalizing risk in osteoporosis management

Personalized risk assessment tools, such as the Garvan Fracture Risk Calculator and the FRAX model, offer a more tailored approach to understanding fracture risk [17]. These tools evaluate multiple factors, including both genetic and environmental influences, such as age, bone mineral density (BMD), fracture history, and lifestyle factors like smoking and alcohol use. Recent developments in genetic research, specifically the introduction of polygenic risk scores (PRS), have further enhanced the ability to predict fracture risk by analysing genetic markers linked to osteoporosis [18, 19]. By combining genomic data with traditional risk factors, it is possible to identify high-risk individuals for early intervention and prevention. These measures could help reduce both the clinical and economic burden of osteoporotic fractures.

1.2 Consequences of fracture

1.2.1 Mortality

Numerous studies have confirmed a significant association between fractures and increased mortality rates, particularly in patients with hip fractures. The risk of death within the first year after a fracture has been reported to escalate from 17% to 36% [20, 21]. The Dubbo study in Australia, one of the pioneering research initiatives on post-major osteoporotic fracture mortality rates, confirmed a marked increase in mortality across all major fractures, especially within a five-year period for both women and men [22]. Their data revealed that the mortality ratio increased by 2.18 times (95% confidence interval [CI], 2.03-2.32) for major proximal femur fractures in women and 3.17 times (95% CI, 2.90-3.44) in men. This higher mortality rate in men may be linked to the greater likelihood of comorbidities at the time of fracture, making them more vulnerable and leading to a higher risk of mortality [22]. Similarly, the mortality ratio rose by 1.66 and 2.38 times for major vertebral fractures in women and men, respectively. Significant increases in mortality rates were also observed for other major fractures, excluding femur and vertebral fractures, with an increase of 1.92 in women and 2.22 in men [22].

A cohort study conducted in Canada, observing 7,753 participants aged 50 years and older, yielded similar results [23]. Their data indicated that individuals who experienced a vertebral fracture within the second year of a five-year follow-up period faced a 2.7 times higher risk of death compared to those without a fracture (95% CI, 1.1-6.6). Meanwhile, participants with hip fractures exhibited a substantially higher risk of mortality, with a hazard ratio [HR] of 3.2 (95% CI, 1.4-7.4). Extended periods of immobility due to hip fractures can lead to several serious medical complications, such as pulmonary embolism, infections, and heart failure [24]. Furthermore, patients with hip fractures often require surgical intervention, which adds additional risks for elderly individuals with pre-existing health conditions [25]. Additionally, the risk of mortality associated with vertebral fractures appeared to be higher in the first year compared to the second year among the female population, with an adjusted hazard ratio of 3.7 (95% CI, 1.1-12.8) and 3.2 (95% CI, 1.2-8.1), respectively [23].

Tran et al. (2018) conducted another cohort study involving 21,123 women and 9,481 men aged 50 years and above in the Danish population. The study revealed that experiencing any form of fracture, including those of the femur, pelvis, vertebrae, or rib, elevated the mortality risk in both men and women [26]. The highest excess mortality rates, observed within one year after the fracture, were associated with hip fractures—33% in men and 20% in women. Furthermore, femur or pelvis fractures were linked to a 20-25% mortality risk, vertebral fractures to a 10% risk, and fractures of the humerus, rib, or clavicle to a 5-10% risk (Figure 1.3). These studies affirm a strong correlation between osteoporosis-related fractures and an increased risk of mortality.

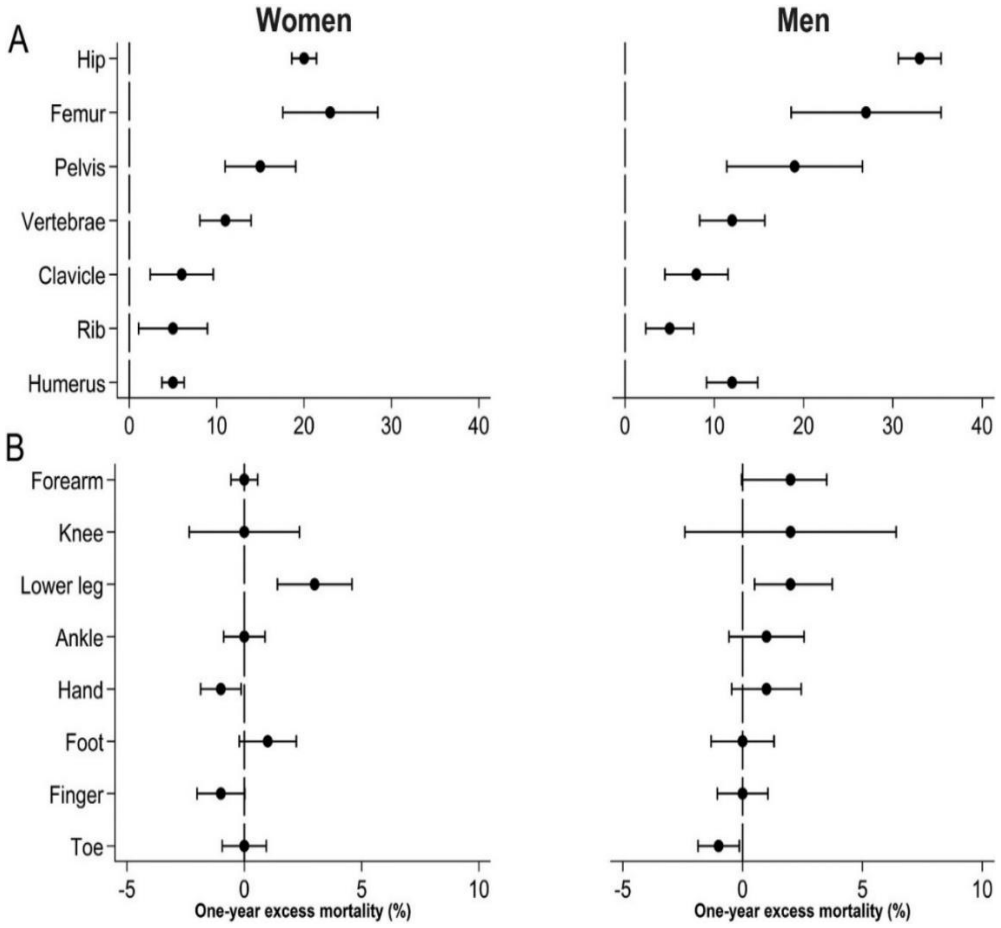

Figure 1.3 Excess mortality after different sites of fragility fracture.

A: hip, femur, pelvis, and vertebral fractures; B: clavicle, rib, humerus, and lower leg fractures. Source: Tran el al., 2018

1.2.2 Morbidity

Besides the risk of death, morbidity is another serious outcome of osteoporosis that should be considered. A cohort study of 10,000 women at the age of 50 suggested that 6.7% of older women who survived osteoporotic fractures will need help for their daily fundamental activities, and approximately 7.8% of patients will require an average of 7.6 years of nursing home care [27]. Besides a cost of approximately US$26,000 per year for hip fracture treatment, patients with hip fractures also had difficulty moving around without walking aids within the first year of recovery [28]. The percentage of patients with femoral fractures who required walking aids for movement also increased from only 40% of patients pre-fracture to 74% of patients who required sticks, and 23% required a walking frame after femoral fractures [29]. Similarly, the percentage of patients who were housebound also increased from 28% pre-fracture to 46% post-fracture [29].

A cohort study of 2,806 patients aged 65 and older revealed that among 120 survivors of hip fractures, there was a slight improvement in mobility noted six months post-fracture. Specifically, 49% were able to dress, 32% could transfer independently, 15% could climb a flight of stairs, and 6% could walk one-half mile [30]. Similar findings were corroborated by data from Mossey et al. (1989), examining hip fracture patients aged 59 and above. This study showcased a decline in the number of patients capable of independent walking, dropping from 81% prior to the fracture to just 21% within 12 months post-fracture [31].

1.2.3 Subsequent fracture

Fractures significantly increase the risk of future fractures, a phenomenon known as subsequent fracture risk. A history of previous fractures is one of the strongest indicators of future fracture risk. A longitudinal study by Center et al. (2007), examining more than 4,000 men and women aged 60 years and older in Australia, found that both genders across multiple age groups experienced increased risks of subsequent fractures following an initial low-trauma fracture [22]. The location of the initial fracture plays a critical role in determining future fracture patterns. For instance, younger men who sustained an initial hip

fracture exhibited nearly a tenfold higher risk of subsequent fractures (RR 9.97, 95% CI 1.38-71.94), while those with clinical vertebral fractures faced a significant risk increase of 15-fold (RR 15.12, 95% CI 6.06-37.65). Moreover, even fractures traditionally considered less severe, such as ankle or rib fractures, were found to significantly increase the likelihood of future fractures—though these effects differed by sex. Ankle fractures were associated with an increased risk in men (RR 4.58, 95% CI 2.44-8.60), but not in women (RR 0.84, 95% CI 0.40-1.76). Conversely, rib fractures increased subsequent fracture risk in women (RR 1.83, 95% CI 1.10-3.04), but not in men (RR 1.30, 95% CI 0.62-2.76). Overall, the relative risk of subsequent fractures was nearly doubled for women (RR 1.95, 95% CI 1.70-2.25) and more than tripled for men (RR 3.47, 95% CI 2.68-4.48), with the increased risk lasting for up to 10 years post-initial fracture [22].

Similarly, Black et al. (1999) demonstrated that vertebral deformities are a strong predictor not only for future vertebral fractures but also for non-vertebral fractures [32]. In their study of women aged 65 and older, those with a history of vertebral deformities had a fivefold increased risk of subsequent vertebral fractures. The increased risk also extended beyond the original fracture site, with a 2.8-fold higher risk of hip fractures and a 1.9-fold higher risk of non-vertebral fractures, indicating that an initial vertebral fracture signals vulnerability across the entire skeleton, not just at the initial fracture site.

This pattern was further confirmed in a study by Hodsman et al. (2008), which found that postmenopausal women in Canada with a history of wrist fractures had a 58% higher risk of experiencing subsequent wrist fractures (95% CI 1.29-1.93) compared to those without a prior wrist fracture [33]. Additionally, Lyles et al. (2008) examined nursing home residents in the United States and found that 23.9% of those with a history of hip fractures experienced subsequent fractures within two years. [34]. The risk of a subsequent fracture was 1.8 times higher in those with prior non-hip fractures (HR 1.84, 95% CI 1.50-2.25) and three times higher in those with previous hip fractures (HR 2.99, 95% CI 2.78-3.21) [34]. This reinforces the idea that previous fractures, regardless of location, substantially raise the likelihood of future fractures.

1.2.4 Quality of life

Quality of life is also reduced significantly with osteoporosis, where patients with osteoporosis-related fractures face pain, disability, and loss of independence. Vertebral deformities are crucial and common outcomes of osteoporosis, where one in four white women had at least one vertebral deformity after menopause [35]. A study involving a population of 2,992 white women aged between 65 and 70 found that women with vertebral deformities had a 1.9 times higher risk of severe back pain (95% CI, 1.5-2.4) compared to women without vertebral deformities [36]. Additionally, vertebral deformities increased the risk of back-related disability by 2.6 times (95% CI, 1.7-3.9), and patients were likely to experience a loss of four or more centimeters in height [36]. Besides the profound effect of pain from a fracture site, women with vertebral fractures also faced disruption related to their emotions with the disability, besides disruption of daily activities [37]. One of the most concerning issues that fracture patients experienced was that pain with movement also increased fear of falling and additional fractures [37].

A significant reduction in quality of life in patients with femoral fractures was observed. The percentage of patients who could do their shopping dropped from 54% before the fracture to only 33% one year post fracture [29]. Besides strong dependence on walking aids due to impaired walking ability, hip fracture patients also showed a clear drop in independence in their level of physical activities of daily living (PADLs), such as eating, bathing, toileting, dressing and grooming, and instrumental activities of daily living (IADLs) such as shopping, preparing meals, doing housework, and prolonged walking distance [38]. Only 56.1% of hip-fractured patients recovered their PADLs, and 37.9% of patients could regain their overall IADLs one year after hospital discharge [38]. Another study from Magaziner et al. (1990) confirmed a similar result when they evaluated the recovery rate of 536 patients aged 65 and older admitted to the hospital with hip fractures and survived after one year of hospital discharge. The study found that most patients could only regain their normal PADLs and IADLs after six months [39], and approximately 17% of patients had to spend the rest of their lives in nursing facilities due to hip fractures [40].

1.3 Physiology and Pathophysiology of Bone

1.3.1 Bone cells

Three major cell types are presented in bone: osteoblasts, osteoclasts, and osteocytes. The osteoblast is from bone marrow-derived stromal cells with a cuboid shape and a size of 20-30μm in diameter. Although osteoblasts have about three months of lifespan and account for only 4-6% of bone tissue, they play an essential role in building bone by depositing the extracellular matrix and performing its mineralization [41].

In contrast, osteoclasts differ from osteoblasts since they arise from hematopoietic stem cells. Osteoclasts are larger, ranging from 10 to 300μm in diameter, and possess multiple nuclei, sometimes up to 100 nuclei [42]. Their lifespan is approximately two weeks, during which they are engaged in the resorption of calcified bone and cartilage, breaking down and removing old or damaged bone tissue that has become mineralized or calcified.

Osteocytes, the most abundant bone cells, constitute 90-95% of all bone cells and are much smaller, measuring 5-7 μm in diameter [41]. Osteocytes originate from mature osteoblasts that have become embedded within the bone matrix during the deposition process [43]. Far from being inactive, osteocytes play a crucial role in bone homeostasis. These cells are embedded within the bone and communicate with other bone cells through an extensive network of interconnecting canaliculi. This network facilitates the transport of small signalling molecules such as prostaglandins and nitric oxide, enabling osteocytes to sense mechanical strain and respond to biochemical signals [44]. Through this communication, osteocytes help regulate bone formation and resorption, coordinating bone remodeling [45]. Additionally, osteocytes have been shown to inhibit dexamethasone-induced apoptosis, which helps in maintaining bone integrity by preventing osteocyte apoptosis [46].

1.3.2 Bone modeling and remodeling

1.3.2.1 Bone modeling

Throughout its lifespan, the human skeleton undergoes two primary processes: modeling and remodeling. The modeling process is significant for skeletal growth, particularly prominent during childhood and adolescence. During this phase, bones alter their shape, size, or positioning under the influence of physiological and mechanical forces [47]. These changes take place on specific bone surfaces, where osteoblasts and osteoclasts respond to biomechanical forces, causing bones to widen or modify their orientation. Wolff's Law describes how long bones adapt their shape in response to the stresses placed upon them [47].

Bone modeling involves the deposition or removal of bone from its surfaces through the actions of formation or resorption. In contrast, bone remodeling is a lifelong process characterized by a coordinated sequence of bone resorption and formation. While this process does not result in noticeable changes to the overall size or shape of bones in healthy individuals, it is crucial for maintaining bone strength by replacing older, microdamaged bone with newer bone [48, 49]. Over time, particularly with aging, the balance between bone resorption and formation can shift. This imbalance can lead to the thinning of cortical and trabecular bone, which weakens the microarchitecture and increases the risk of fractures or bone collapse [50]. Therefore, while the overall shape of the bone may remain unchanged, its internal structure can become compromised, leading to increased fragility. While bone remodeling occurs more frequently in adults compared to bone modeling, its processes focus on the maturation, maintenance, and mineral metabolism of bone [51].

1.3.2.2 Bone remodeling

Bone remodelling is an ongoing process of bone resorption and formation throughout the skeleton, which relies heavily on the balance between osteoclasts for bone resorption and osteoblasts for bone deposition, both in time and space (Figure 1.4) [15, 52]. Remodelling

occurs in response to specific stimuli, either targeting injured areas that require repair, such as micro-fractures (referred to as targeted remodeling) [48, 53], or addressing broader physiological needs such as maintaining mineral homeostasis by accessing calcium and phosphate stores (referred to as random remodelling) [54]. Additionally, preferential bone loss occurs at specific sites in response to stimuli like disuse osteoporosis, glucocorticoid-induced osteoporosis, hypogonadism, and hyperparathyroidism, where bone resorption may outpace formation, particularly in trabecular-rich areas like the spine or weight-bearing regions [55]. These factors will be accounted for later in the study by incorporating variables such as medication use and underlying conditions into the data analysis.

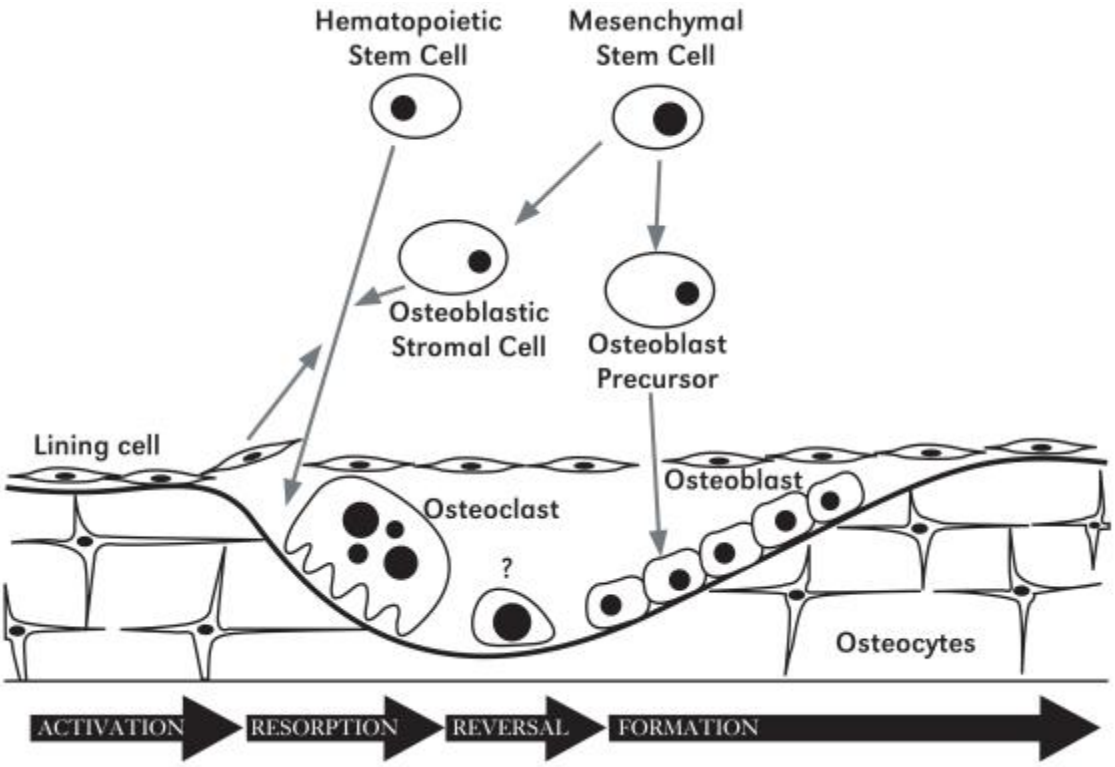

Figure 1.4 Bone remodelling**.** Source: Office of the Surgeon, 2004

A group of bone cells involved in remodelling is called the Bone Remodelling Unit or Basic Multicellular Unit (BMU), which accomplishes the whole process in four sequential phases: activation, resorption, reversal, and formation. In the activation phase, osteocytes and other factors such as Insulin-like Growth Factor 1 (IGF-I), Tumour Necrosis Factor alpha (TNF-α), Parathyroid hormone (PTH), Interleukin-6 (IL-6), and Monocyte Chemoattractant Protein-1 (MCP-1) enter the bone microenvironment to recruit osteoclast precursors to the site of resorption by signalling stromal or lining cells [56]. Next, osteoclasts differentiate, polarize, and adhere to the surface of the bone. This resorption phase lasts 2-3 weeks in humans, during which osteoclasts dissolve the inorganic component of the bone matrix and release lysosomal enzymes to dissolve the organic component [57].

Compared to the resorption phase, the reverse phase is shorter, lasting about nine days, during which osteoclast apoptosis is promoted to stop resorptive activity. Additionally, reversal cells remove debris from the degraded bone matrix and respond to signals from factors released during the previous phase, preparing the bone for the anabolic action of osteoblasts [58] In the formation phase, which takes approximately 4-6 months to complete, osteoblasts arrive at the resorbed area and secrete many molecules to produce a new bone matrix (osteoid) that is initially unmineralized before the process of mineralization concludes the remodeling process. During this phase, a subset of osteoblasts becomes entrapped within the bone matrix, differentiating into osteocytes. Another group of osteoblasts on the bone surface becomes quiescent, flattened lining cells, preventing further interaction between osteoclasts and the bone matrix until the next cycle [59]. Meanwhile, approximately 50-70% of osteoblasts undergo apoptosis during this phase [58].

1.3.3 Bone turnover markers

Data from Riggs and Melton (2002) confirmed that high bone turnover markers (BTMs) during the bone remodeling process increase the risk of fracture. Similarly, a study by Heaney (2003) revealed that reducing bone turnover effectively serves as a therapy for osteoporosis by halting and reversing the loss of bone mass [60-62]. Similarly, the effectiveness of antiresorptive agents on osteoporosis treatment was observed. Based on 12

meta-analyses of clinical trials for fracture intervention, it was found that using antiresorptives for osteoporosis treatment could reduce the risk of fracture by 20% and up to 45% [63]. Using antiresorptive drugs was also effective for improving spine bone mineral density by 16%, which led to a reduction in fracture risk, confirming the significant role of BTMs both in bone remodelling and in osteoporosis. In addition, bone turnover markers are classified into two groups: bone resorption markers and bone formation markers.

1.3.3.1 Markers of bone resorption

The N-terminal telopeptide (NTX) or Amino-terminal cross-linking telopeptide of type I collagen is the most widely used marker for bone resorption. NTX can be measured in either serum or urine since it is the breakdown product of type 1 collagen. From an observation of a placebo-controlled trial in Japan, the results indicated that the amount of serum NTX and urinary NTX in postmenopausal women under osteoporosis treatment showed a significant reduction after antiresorptive therapies compared to the control group [64]. However, the reduction in NTX measurement in urine could be detectable clearly after four weeks, while serum NTX is less sensitive and had lower efficacy when showing precise results until 16 weeks [64].

Carboxyl-terminal cross-linking telopeptides of type I collagen (CTX) is another bone resorption marker similar to NTX, which can be detected in both serum and urine. CTX derived from the α1 chain of human type I collagen (α CTX) originates mainly from bone and represents the breakdown product of synthesized collagen, which then undergoes β-isomerization (βCTX) as a representation of aged collagen since it increases with aging [65]. A study by Garnero et al. (2002) confirmed that postmenopausal women with a higher ratio of αCTX/βCTX had a higher risk of bone fracture than those with a lower ratio [66]). Therefore, the ratio of αCTX/βCTX can be an indicator for diagnosing bone disease.

Other makers offer a reliable assessment of bone resorption, such as pyridinoline (PYD) and deoxypyridinoline (DPD) cross-links and Tartrate-resistant acid phosphatase (TRACP5b). Regarding PYD and DPD cross-links, both are produced from collagen breakdown during bone resorption and can be measured in urine. These collagen cross-links

are present in many areas, such as cartilage, ligaments, vessels, and bone for sources of PYD or in bone and dentin for DPD sources [67]. However, most circulating PYD, urinary PYD, and DPD come from bone; therefore, they are reliable resources for bone resorption assessment [67].

Tartrate-resistant acid phosphatase (TRACP5b) is another bone resorption marker and is remarkable as the only maker expressed in osteoclast activity. However, two studies conducted by Halleen et al. (2001) and Hannon et al. (1998) concluded that TRACP5b may not be a reliable indicator for assessing osteoporosis due to its inconsistent sensitivity [68, 69]. Similarly, other makers in bone resorption, such as Carboxy-terminal pyridinoline cross-linked telopeptide of type I collagen (ICTP) and hydroxyproline have been studied but do not have the sensitivity and specificity required to reflect bone resorption, as the concentration in urine can be affected by dietary collagen [70, 71].

1.3.3.2 Markers of bone formation

Several serum markers of bone formation, namely osteocalcin, alkaline phosphatase and bone-specific alkaline phosphatase, procollagen type I N-terminal propeptide (PINP), and procollagen type I C-terminal propeptide (PICP). Firstly, osteocalcin or bone gla protein (BGP) is the non-collagenous bone matrix protein synthesized by mature osteoblasts [72]. Most of the synthesized osteocalcin is involved in bone matrix in the mineralization process, while a small percentage is released into circulation and can be detected by immunoassay, making it a good marker of bone formation [73]. However, osteocalcin in the serum is easily degraded after a short period at room temperature, affecting their reactivity [67].

Similarly, alkaline phosphatase (ALP) is an ectoenzyme on the membrane of osteoblastic cells that is also required for bone mineralization [74]. About half of the circulating ALP in the human body is related to activity in bone. These bone-specific ALPs can be assessed by immunoassays, making them a great indicator for osteoblast function [75]. Next, procollagen type I N-terminal propeptide (PINP) and procollagen type I C-terminal propeptide (PICP) are two peptides that originate mainly from bone that can be used to determine the activation of bone formation based on their products in the circulation [67]

[76]. Despite the unknown functions of these bone formations and the numerous limitations regarding their biological activity and variability, studying these bone turnovers remains a valuable tool for estimating fracture risk [77-79].

1.3.4 Measure of bone mass

1.3.4.1 Bone mineral density

Bone mineral density (BMD) is one of the most critical factors for indicating bone strength and predicting fracture risk. Based on physics, BMD should ideally be measured by the mineral content per unit volume of bone at a specific site. Although high-resolution imaging techniques such as HR-pQCT and central quantitative CT can provide 3D assessments and volumetric BMD measurements, their clinical availability remains limited, and they are not routinely used in standard practice. Instead, dual-energy X-ray absorptiometry (DXA) is commonly used to assess bone density over a 2D surface area, providing measurements of bone mineral content per square centimetre [80, 81]. This means that BMD, as measured by DXA, represents the average concentration of bone mineral per defined area, not per volume, as the term 'density' might suggest. Because DXA is a 2D measurement, it acts as a surrogate measure of bone size, meaning that smaller bones may appear to have lower BMD even when the internal density and structure of the bone remain unchanged [82]. This limitation of DXA is especially important when comparing individuals with different bone sizes, such as women and men.

Dual-energy X-ray absorptiometry (DXA) is considered the gold standard for measuring bone density. According to Watt's study (2004), DXA is a non-invasive method that swiftly and accurately measures bone density by employing two X-ray beams of different energy levels at the target site. Since bone contains minerals that absorb X-rays, DXA estimates bone density by calculating the difference between X-ray input and the amount of X-ray absorption by the bone [83].

1.3.4.2 T-score and Z-score

The measurement of bone mineral density changes with age. Bone mineral density actively increases until it reaches a peak at the age of 20-30, then declines slowly to around the age of 40 and continues decreasing rapidly after menopause, and begins to reduce gradually with age from 60 to 90 years old [84].

T-score. According to World Health Organization, the standardized BMD measurement (BMD T-score) is one of the indicators for risk of fracture based on comparing the BMD of the individual to the standard BMD of young, healthy sex-matched population (pBMD) with a constant standard deviation (SD) as reference.

$$\text{BMD T - score} = (\text{BMD} - \rho\text{BMD})/\ \text{SD}$$

Table 1.1 illustrates the criteria of WHO for using T-score at the femoral neck for diagnosing osteoporosis, where individuals with a T-score at the femoral neck bone mineral density of ≥ -1.0 SD are considered normal and osteoporotic with BMD T-scores of less than -2.5. Furthermore, an individual with osteoporosis (T-score ≤ -2.5) and a history of fracture is considered severely osteoporotic.

Z-score. While the T-score is used to determine the risk of fracture in the aging population, after menopause for women, the Z-score is a valuable measurement based on BMD for evaluating whether the BMD of an individual is reaching their peak bone mass or not in a healthy young population. The Z-score of an individual can be derived as the difference between the BMD of an individual and the average BMD (aBMD) of a healthy individual at the same age to standard deviation of BMD at that age (SD_{aBMD}), where individuals with BMD Z-scores of -1.0 means that their bone density is 1SD lower than the norm for people at that age.

$$\text{BMD Z - score} = (\text{BMD} - \text{aBMD})/\ SD_{aBMD}$$

Table 1.1 Criteria of bone density based on T-score for Osteoporosis

T-score	Classification	
≥ -1.0 SD	Normal	
-2.5 SD < T-score < -1.0 SD	Low bone mass (Osteopenia)	
T-score ≤ 2.5 SD	Osteoporosis	
T-score ≤ 2.5 SD and fragility fracture	Severe osteoporosis	

*Data from Kanis et al., 1994

1.4 Risk Factors for Osteoporosis Fracture

1.4.1 Age

Another important indicator for fracture is advancing age, since there is an increased risk of fractures in the older population compared to the younger population [85]. A study by Cummings et al. (1995) from a population of Caucasian women confirmed that the risk of hip fracture increased by 1.5 times per five years of age [86]. Similarly, the study of Seeley et al. (1991) of 9,704 nonblack women age 65 years or older also showed that the risk of hip fracture increased exponentially from 1.4 per 1000 person-years around age 60 years, to 3.2 to 6.4 per 1000 person-years at 70 to 74 years and 75-79 years and 10.7 per 1000 person-years in patients 80 years or older [87].

Furthermore, the increase in osteoporotic fractures with advancing age happens exponentially regardless of gender or race [88]. The study from Change et al. (2004) also indicated a clear relationship between fracture risk with aging despite gender by observing a cohort of 16,473 men and 22,884 women in Australia. Their data shows only 116 cases of hip fracture in women and 0 cases in men per 100,000 person-years in the age group of 60-64 years, while the cases of hip fracture increased to 2,597 in women and 1,187 in men per 100,000 person-years at the age of 85 or older [89].

However, the relationship between fracture risk and advancing age is not strong in all fracture types. Nguyen et al. (2001) found that the risk of fracture for the humerus increased by 2.8, whereas the risk of fracture for the forearm increased 1.6-fold in men with every five additive years of age. In addition, there was a steep increase in fracture risk in the hip and

proximal humerus. In contrast, the fracture risk in vertebrae was moderately steep and had no increase for distal forearm and foot sites, associated with increasing age in women 65 years or older [90].

1.4.2 Gender

Gender is an important factor that strongly correlates with the risk of fracture because of a distinction in fracture rates between women and men (Figure 1.5) [13]. There was a clear difference in the number of fractures by gender [91]. The study by Nguyen et al. (2008) of a population of 1,358 women and 858 men aged 60 years or older found that the incidence of fracture in women was much higher than in men, with 426 cases making the overall incidence of fracture 35 per 1,000 person-years while only 149 cases in men (18 per 1,000 person-years) [91]. Another study by Johnell et al. (2005) showed a similar trend in which the risk of fracture for women over their lifetime was from 40% to 50%, but the percentage of fracture in men is only between 13% and 22% within the same age group [9].

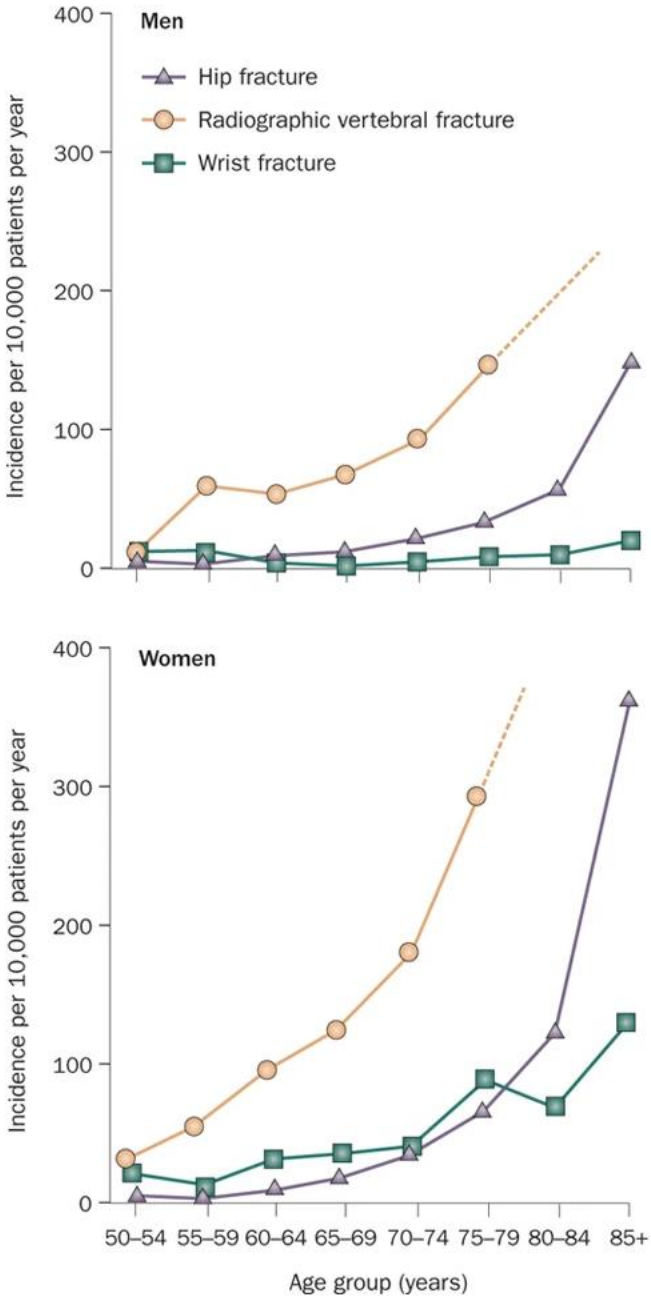

Figure 1.5. The incidence of radiographically determined vertebral, hip and wrist fracture by age and gender. Source: Harvey et al., 2010

The research of Kanis (1993) stated that women have a higher risk of hip fracture than men by six-fold, because women have lower peak bone density during maturity, accelerated bone loss after menopause, and a longer lifespan than men [92]. Furthermore, cortical bone loss is more significant and begins in the middle of a woman's life, after their menopause. In contrast, the loss of cortical bone in men happens significantly after age 75 [93]. Additionally, estrogen deficiency after menopause in women increases bone remodelling activities, with a shift toward resorption rather than the formation of new bone [94]. This imbalance not only accelerates bone loss but also leads to changes in the bone microarchitecture, resulting in the thinning of both cortical and trabecular bone, which compromises bone strength and increases the risk of fractures [81].

Both older women and men from 67 to 69-year-old showed similar increments in bone loss, ranging from 1.8-6% per 10 years in different skeletal sites such as the lumbar spine, proximal femur, and femoral shaft. However, after menopause, women experience a much greater decline in bone mass compared to men of the same age, with the rate of bone loss estimated to be two to five times higher in women [95]. Sigurdsson et al. (2006) observed that the cross-sectional area of bone in women's vertebrae, the femoral neck, and the femoral shaft was smaller than in men by 24.9-31.7% [95]. This difference in bone size contributes to lower BMD readings in women when using DXA, as DXA is a surrogate measure that reflects bone size rather than directly assessing internal bone structure. Even if women have similar internal bone density and microarchitecture as men, their smaller bone size can result in lower BMD values, contributing to a higher fracture risk [96].

1.4.3 Bone mineral density

Many previous studies have confirmed a significant negative relationship between BMD and risk of fracture in which each reduction of BMD T-score increases the risk of fracture by 2 to 3-fold [97-99]. According to a study by Nguyen et al. (1993) on a population aged 60 or older, each standard deviation lower in femoral BMD was associated with a 2.4 fold (95% CI 1.92-2.97 with SD=0.13 g/cm^2) increase in the risk of fracture for women and 2.0 fold (95% CI 1.51-2.61 with SD=0.15 g/cm^2) increase in the risk of fracture for men [99].

While Marshall et al. (1996) reviewed studies of 11 cohorts from around the world, they found that a 1 SD decrease in bone density at the femoral neck increased the odds for fracture by 2.7 in an average of 8 study populations shown in Table 1.2 [97].

Furthermore, the association between BMD and fracture risk is site-dependent [97]. Based on the measurement of BMD in the hip, each 1SD decrease in BMD will increase the risk of hip fracture by 2.6 times, forearm fracture by 40%, vertebrae by 80%, and 60% for all fracture types (Table 1.3). However, based on the BMD measurement in the lumbar spine, the odds ratio of fracture for 1SD decrease is different, increasing the risk of hip fracture by 60%, forearm fracture by 50%, vertebrae by 2.3 times, and all types of fracture by 50% [97]. Since the association of decreasing BMD with fractures is higher in the hip compared with other sites, BMD measurement in the hip location is considered the first choice for observation shown in Table 1.3 [97].

Table 1.2 Summary of case-control studies of hip fracture in women

Study	Odds ratio of fracture for 1SD decrease in BMD at femoral neck
Greenspan et al.1994	2.0
Sugimoto et al.1994	2.5
Karlsson et al.1993	1.5
Kanbera et al.1992	3.3
Libanati et al.1992	9.0
Nakamura et al.1992	1.5
Perloff et al. 1991	3.0
Chevalley et al.1991	3.3
Average	2.68

Adapted from Marshall et al., 1996

Table 1.3 Summary of meta-analysis: relative risk (95% confidence interval) of fracture for 1SD decease in bone density below age adjusted mean

Site of BMD	Forearm fracture	Vertebral fracture	Hip fracture	All fracture
Hip	1.4 (1.3-1.6)	1.8 (1.1-2.7)	2.6 (2.0-3.5)	1.6 (1.4-1.8)
Lumbar spine	1.5 (1.3-1.8)	2.3 (1.9-2.8)	1.6 (1.2-2.2)	1.5 (1.4-1.7)

Adapted from Marshall et al., 1996

1.4.4 Low body mass index

Bone mass index (BMI) measures body fat by an individual/s weight in kilograms divided by their height in meters squared (kg/m ^{2}). According to World Health Organization, a BMI of 18.5 to 24.9 is considered normal weight; lower or higher than that is classified as underweight (BMI<18.5) or overweight (BMD ≥ 25) [100]. Several studies have shown an association of low BMI with low BMD and fracture. Women with osteoporosis had lower BMI (23.7kg/m ^{2}) than individuals with normal BMD (28.5 kg/m^2), and the risk of fracture increased by 8% for each one-unit decrease in BMI [101, 102].

Considering a BMI of 25 kg/m^2 as standard, people with a lower BMI (20 kg/m^2) had an increased risk of experiencing hip fracture by nearly two-fold (RR=1.95; 95% CI, 1.71–2.22), while the risk of hip fracture was decreased by 17% for people with BMI of 30 kg/m^2[103]. The risk ratio (RR) of osteoporosis fracture changed depending on BMI in men and women based on observation of 12 population-based cohorts [103]. In women, people with low BMI had a higher risk of osteoporosis than women with a normal range of BMI [104]. Among 1,769 women participants from 50 to 84 years old, the risk ratio for osteoporosis increased 76% for women with low BMI (95% CI 1.2- 2.7), decreased 54% for women with high BMI 0.46 (95% CI 0.29-0.71), and decreased up to 78% for women with obese BMI (≥30) (95% CI 0.14- 0.36) [104]. Although the findings of Nitzan-Kaluski (2003) reported a higher risk (decreased by 12%) with each one-unit increase in BMI, both studies confirmed a significant association between BMI and osteoporosis [101, 104].

1.5 Lifestyle factors

1.5.1 Smoking

Among a population of more than 9,000 Caucasian women, Cummings et al. (1995) found that current smokers had a higher risk of fracture, increased by two-fold compared with a non-smoker [86]. A meta-analysis by Kanis et al. (2005) of three cohort studies confirmed similar results, that an individual who currently smokes had a 60% higher risk of hip fracture (95%CI, 1.27–2.02) after adjusting for age and BMD, or increased 84% (95% CI, 1.52–2.22) in the same site with age and no BMD adjustment [105]. Furthermore, the effect of smoking on the risk of fracture is much higher in men than in women, with 59% and 36%, respectively [106].

Besides the significant correlation between smokers and non-smokers to the risk of fracture, the fracture ratio is also different depending on cigarette consumption and fracture sites [107, 108]. The risk of hip fracture in women from 34 to 59-year-old increased by 2-fold to 60% (95% CI, 1.1-2.3) when smokers consumed more than 25 cigarettes per day, compared to 30% (95%CI, 1.0-1.7) in smokers with lower cigarette consumption [107]. Furthermore, the risk of hip fracture in smokers was temporal as the risk ratio of fracture in former smokers reduced to 0.7 (95%CI, 0.6-0.9) after ten years of cessation compared with current smokers [107].

Baron et al. found a similar result where current smokers had a higher risk of hip fracture than non-smokers, by 66%, while former smokers had only a 15% increase in fracture risk [109]. However, the risk of fracture of the individual was also dependent on the duration of smoking instead of the number of cigarettes smoked because the fracture risk was not consistent with the number of cigarettes. The risk of hip fracture was increased by 36% with 1-10 cigarettes per day, by 51% with 11-20 cigarettes per day, but only about 22% for more than 20 cigarettes per day; while the duration of smoking showed a clear correlation by increasing 6% (95% CI, 0-13%) of fracture risk in the hip for every five years of smoking with age-adjusted [109].

1.5.2 Alcohol

Similar to smoking, alcohol is also considered a factor causing osteoporosis fracture. Consuming alcohol more than two units per day increased the hip fracture by 68% (95% CI, 1.19–2.36) after adjusting for age, it increased up to 70% (95% CI, 1.20–2.42) with age and BMD adjusted (Kanis et al., 2005). Another study from Høidrup (1999) stated similar results in terms of the impact of alcohol consumption on fracture risk. They found that male drinkers who consumed more than 27 drinks per week increased the risk of fracture by 75% compared with non-drinkers [110]. However, the thresholds of harmful drinking toward hip fracture are different between men and women since the risk of hip fracture in women was increased by 44% when consuming 14-27 drinks per week but only increased by 12% when women drank more than 28 drinks per week. Furthermore, the risk of hip fracture in men was observed in different levels of alcohol intake and increased based on their alcohol intake [110]. While women saw a significant increase in the risk of hip fracture with 14-27 drinks per week, men needed to drink more than 27 drinks (28 to 41 drinks/week) for association with hip fracture. In addition, male drinkers increased their risk of hip fracture by 1.75 (95% CI 1.06-2.89) with 28 to 41 drinks/week, 1.95 (95%CI 1.06-2.89) with 42-69 drinks/week, and by 5.47 (95% CI 2.60-10.70) with 70 drinks or more [110].

1.5.3 Physical activity

Physical activity is one environmental factor that strongly affects fracture risk in the aging population by increasing skeletal strength and improving muscle mass [111, 112]. Older people of both sexes had a lower risk of fracture by increasing their daily activities such as standing, walking, or gardening [113]. Compared to women and men who were standing more than 60 minutes per day, women with only 30 minutes of standing time per day increased the risk of hip fracture by 80%, while the risk was increased by 90% in men after adjusted BMI and other factors [113].

A similar study from Cummings et al. (1995) showed a clear association between daily activities and reduced hip fracture, where they noticed that women who used their feet

for physical activities for about four hours or less each day increased the risk of hip fracture by two times than those who spent more times than four hours each day [86]. Furthermore, the fracture risk decreased by 10% (RR, 0.9; 95% CI, 0.8-1.0) for every five blocks walked daily [86]. From a study of 61,200 women from the age of 40 to 77 years, Feskanich et al. (2002) found that women spending 4 hours or more per week walking could reduce the risk of hip fracture by 41% (RR, 0.59; 95% CI, 0.37-.094) compared to those who did no exercise or walked less than one hour per week [114].

1.5.4 Calcium intake

Calcium is essential in the human body, especially bone health since more than 95% of calcium is in bones and teeth [115]. Based on the close link between calcium and bone, many studies focus on the impact of calcium on fracture risk. According to a study by Black et al. (1996) in women aged 55 to 81, 82% of osteoporosis patients had lower calcium intake than recommended of 1000mg daily [116]. Another study among the Yugoslavian population with different levels of calcium intake showed that people with lower calcium intake had a higher incidence rate for hip fracture [117]. Similarly, postmenopausal osteoporosis patients treated with calcium alone or combined with vitamin D, oestrogen, or fluoride had a lower rate of fractures than those who were not taking supplementation [118].

However, the association between calcium intake and fracture risk was not consistent and significant between men and women. A meta-analysis of 6 cohort studies with a population of 170,991 women by Bischoff-Ferrari et al. (2007) found that there was no relationship between 300 mg total calcium intake daily and risk of hip fracture, where the risk ratio was 1.01 (95% CI, 0.97-1.05), while study among 68,606 men from five different cohort studies showed a pooled risk ratio of 0.92 (95% CI, 0.82-1.03) for 300 mg of calcium per day [119]. On the other hand, there was a relationship between the risk of fracture and calcium intake in elderly British men who took more than 1g of calcium per day, which has a protective effect for hip fracture. However, this relationship was not found in women [113].

Additionally, women had no apparent association or negative relationship between calcium and fracture. Cumming et al. (1997) found from an observation of 9,704 elderly

white women from the age of 65 years or older, that the risk of hip fracture was increased by 1.5 (95% CI, 1.1-2.0), and the risk of vertebral fractures increased by 1.4 (95% CI, 1.1-1.9) for women who currently took calcium supplements [120].

1.6 Osteoporosis as a genetic disease

Besides many environmental factors such as smoking, alcohol, calcium intake, or physical activities, many studies also confirmed the relationship of genetic factors based on twins or family history of fracture, suggesting certain gene polymorphisms can affect fracture or risk of fracture [121, 122].

1.6.1 Heritability of bone mineral density

Bone density is an essential factor in identifying the risk of osteoporotic fracture. Therefore, the genetic variants involved in bone density have an essential role in determining candidates for many genome-wide association studies [123, 124]. Based on data from a polymerase-chain-reaction-based assay in a Dutch population of 1,778 women after menopause, there was a significant relationship between *COLIA1* polymorphisms and bone density in hip and spine sites [125]. The study identified that women with the *SS* genotype had the highest value of bone density compared with the genotype of *Ss* and *ss*, which had an intermediate and the lowest bone density, respectively. A similar study was conducted by Langdahl et al. (1998) investigating the association between the *COLIA1* gene and osteoporosis in fractured populations [126]. They found that participants with *ss* genotypes had lower BMD in hip and spine sites than *SS* and *Ss* genotypes [126]. Furthermore, the *ss* genotype appeared more frequently in the fractured group (14.3%) than the control group (1.4%) [126].

The heritability of BMD is higher at the lumbar spine compared to other skeletal sites like femoral neck and distal radius (Figure 1.6)[127]. In family studies, there was evidence for the inheritance of BMD from family members from which the heritability of BMD in the lumbar spine was from 61-67% and about 44-76% for BMD of the femoral neck [128]. When looking at the genetic contribution between children and parents, there was a significant

correlation in BMD of lumbar and femoral areas between mother and daughter pairs [129]. For instance, mothers with low bone mineral content (BMC) of the radius tended to have a daughter with low BMC [130]**.** Similarly, other family studies found similar results, where the BMD of the hip and femoral neck of daughters who have a mother with osteoporosis fractures or low BMD, were lower by 5-8% than those who had a mother with normal BMD, and the heritability of BMD among daughters was estimated to be from 34-63% [131].

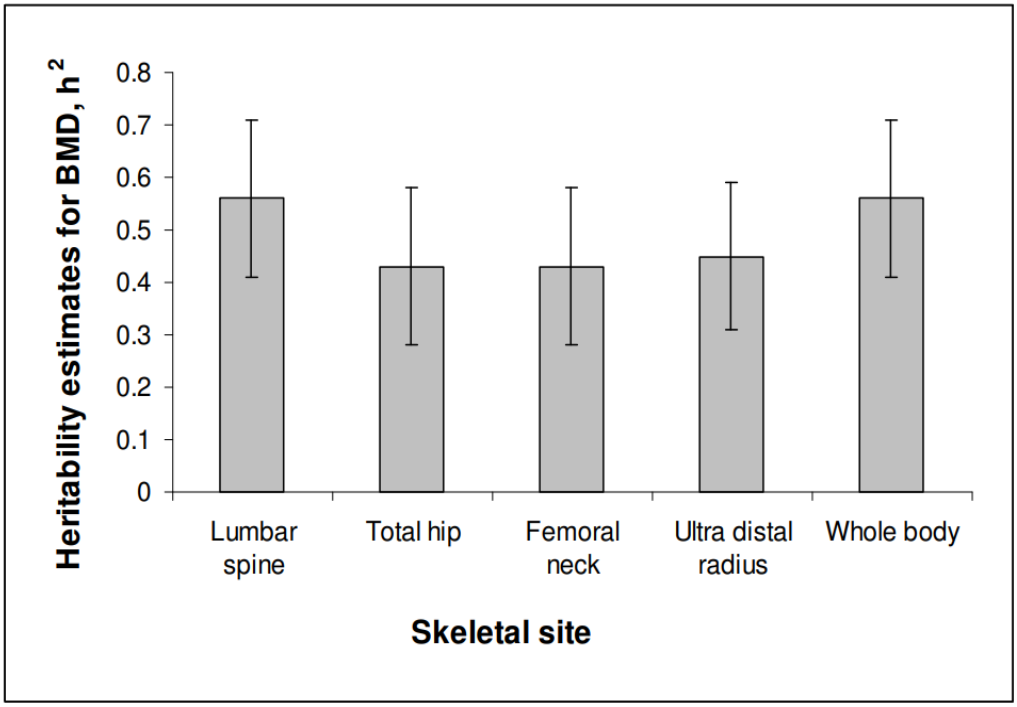

Figure 1.6 Estimates of the heritability for BMD. Source: Lenchik et al., 2004.

Twin studies are also another way to confirm the heritability of BMD as a genetic factor to indicate osteoporosis. The correlation of BMD in the spine and proximal femur in monozygotic twins was more similar than pairs of dizygotic twins since monozygotic twins have more common alleles than dizygotic twins [132]. According to a study of 724 female twins, Makovey et al. (2007) found that the genetic factors accounted for the rate of bone loss per year differently depending on sites, with 38% in the lumbar spine, 49% in the total forearm, and about 44% for the whole body [133].

1.6.2 Heritability of fracture

An observational study of 6,570 UK female twins, where wrist fractures accounted for 3.3% of the population, revealed a case-wise concordance of 0.28 in monozygotic twins (MZ) and a lower concordance of 0.11 in dizygotic twins (DZ) [134]. These findings confirmed a heritability of 54% in wrist fracture liability, irrespective of age considerations [134].

1.6.3 Collagen type I α1 gene (COLIA1)

The collagen type I α1 gene (*COLIA1*) belongs to the collagen superfamily as one of the most important protein sources in bone [135]. Previous studies identified an association of *G* to *T* polymorphism in the regulatory region for specificity protein 1 (Sp1) binding site in *COLIA1* with BMD [136] and osteoporotic fracture in several populations [126, 137]. According to Harris et al. (2000), there was an association of *COLIA1* polymorphism with bone loss in their observation of 243 men and women aged 65 years and older [138]. Although there was no significant difference in the baseline BMD within the three genotypes, participants with the *ss* genotype had an increased rate of bone loss in the total body by 9.4% than participants with the *SS* genotype [138]. However, there was no observation of a significant relationship between *COLIA1* polymorphism with bone loss at the femoral neck and lumbar spine. Another study on *COLIA1* was conducted by Macdonald et al. (2001) to examine the effect of *COLIA1* polymorphism on bone loss. The study observed a population-based cohort of 734 perimenopausal and early postmenopausal women in Scotland. While

there was no association between *COLIA1* polymorphism and bone loss in HRT users, women without HRT had a higher rate of bone loss at both the lumbar spine and femoral neck when they carried the *ss* genotype [139].

In contrast, Yazdanpanah et al. (2007) reported the opposite effect in study on 6,280 postmenopausal women in Rotterdam [140]. After 7.4 years of follow-up, they found no association between the *COLIA1* genotype and change in femoral neck BMD. However, women with a homozygous recessive allele (*TT*) had a higher risk of fracture by 2.3 times than those with a homozygous dominant allele (*GG*) [140].

1.7 Genome-wide association studies

Genome-wide association studies (GWAS) or whole-genome association studies investigate an association between a large set of genetic variants with up to 1 million single nucleotide polymorphisms (SNPs) and a heritable trait or disease risk in a population [141]. Compared to linkage analyses, association studies are becoming more popular as they have several advantages. Linkage analyses were used to observe the differentiation of genetic markers and traits among related individuals to identify quantitative trait loci. Furthermore, linkage analysis can only investigate small regions of one or two chromosomes, and these regions were chosen based on the findings of other studies. On the other hand, GWAS can be used to study unrelated individuals, and the regions size can be from small to the whole genome that is hypothesis-free (Figure 1.7). Therefore, GWAS has become one of the popular approaches to identifying the genetic traits of osteoporosis studies.

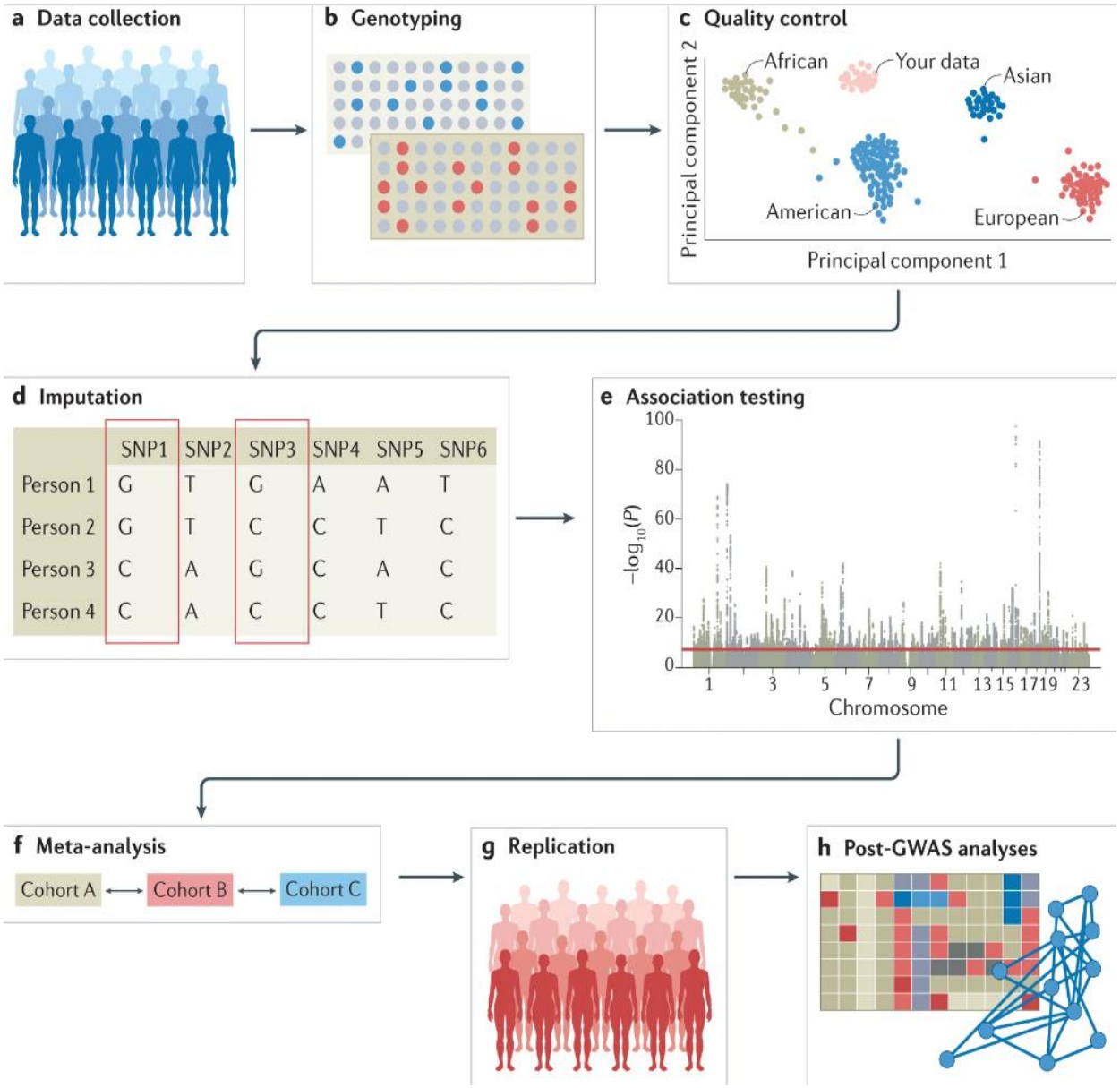

Figure 1.7 Overview of steps for conducting GWAS**.** Source: Uffelmann et al.,2021

1.7.1 GWAS and bone loss

While there are numerous GWAS on bone mineral density as one of the primary risk factors of osteoporosis and fractures, the GWAS studies focusing on discovering the genetic traits affecting the variation of rate in BMD are limited [142]. First of all, BMD was chosen as the primary trait in many GWAS because it has high heritability between-individuals, with more than 50% [132, 143]. In addition to that, there is a significant association between BMD and fractures that has been confirmed in many studies [144]. The most crucial factor is that there is availability of large cohorts for conducting research on BMD compared to other traits [145-147]. Conversely, studying bone loss is a complex phenotype that necessitates serial measurements of BMD to track changes over time [142]. Consequently, there hasn't been a GWAS specifically dedicated to bone loss. Nevertheless, various genes identified through GWAS of cross-sectional BMD serve as potential candidate genes associated with bone loss.

These genes are expected to include members of the beta-catenin/Wnt signalling pathway. The Wnt pathway was confirmed to play an essential role in bone formation with osteoblast genesis, osteoblast proliferation, and apoptosis of osteoblasts and osteoclasts [58, 148]. According to the study of Gong et al. (2001), low density lipoprotein receptor-related protein 5 (LRP5) from the Wnt signalling pathway worked as a regulator for bone-mass accrual. Since its mutation led to a decrease in bone thickness and can cause osteoporosis pseudoglioma [149], other studies were conducted and found that LRP5 and its mutation were significantly associated with bone mass [150, 151]. Other potential genes for bone loss are related to the RANKL/RANK/OPG pathway, which regulates the relationship between osteoblast and osteoclast activity in bone remodeling [152, 153]. The receptor activator of nuclear factor kB (RANK) within this pathway was confirmed to play a crucial role in the regulation of osteoclast formation and differentiation [154]. Next are developmental genes involved in endochondral ossification, such as transcription factors (TFs) and oestrogen endocrine pathways [155, 156]. Many studies found that TFs induce the expression of critical genes in the ossification process by mediating the differentiation of osteoclasts, as well as osteoclast survival and activities [157-159].

1.8 Challenges remaining in genetics research

One challenge in genetics research for osteoporosis is that many genes associated with bone phenotypes have not been studied [160]. Although bone mineral density is considered a vital factor to indicate osteoporosis, measured BMD alone is not an accurate and consistent indicator in all cases, since people who had the exact measurement of BMD still have different fracture risks [161].

In addition, the genome-wide association studies by Estrada et al. (2012) discovered that 14 out of 56 loci for BMD were associated with fracture risk [162]. However, some candidate genes that are essential in bone metabolism, were not confirmed as significant in their studies, such as asopressin (*Avp*), oxytocin (*Oxt*), and β-2-microglobulin (*B2m*) which function to regulate the formation and/or function of osteoblast and osteoclasts, or β-2-microglobulin (*B2m*) in bone remodeling [147, 163-165]. Therefore, the density of bone alone is not an accurate determination of bone strength and quality. Other different interdependent factors that affect the organization of bone matrix, such as its nature of organic or mineral, its structure with more or fewer osteons in cortices, its arch-like packets in trabecular bone, its texture of woven or lamellar bone, its microarchitecture and its macroachitecture, that lead to a quality of bone and fracture risk [161].

Furthermore, there are no significant associations regarding whether identified genetic variants are related to bone loss. More than 307 genetic variants at 203 loci were associated with BMD of the heel, based on GWAS of 142,487 participants [166]. However, most identified genetic variants had a negligible effect rate on bone loss with an odds ratio from 1.02 to 1.15, making them inaccurate enough for fracture risk assessment [167].

An inconsistent association between BMD-related genetic variants and osteoporosis in humans and other animals is another challenge that researchers have to face. Other studies identified loci associated with bone loss from animal studies, such as on mice, rats, or baboons [168-170]. However, Klein et al. (2004) found that the *alox15* genes in mice had a negative association with BMD, which is in contrast to a study by Mullin et al. (2007), who found *alox15* genes affected BMD in humans [171, 172]. Similarly, the study of Bassett et

al.(2012) among 100 knockout mouse strains discovered nine new genes related to bone mass and strength, while those genes had not yet been found in human studies [173].

More recently, taking advantage of genome-wide association studies, it is possible to test for genetic variants in the population associated with different traits of osteoporosis, such as BMD and fracture [174]). However, most marker sets in GWAS are designed to identify genetic variants with a minor allele that has 5% or greater frequency. Therefore, this approach is not efficient with a small sample size and toward rare polymorphisms with 5% or less population frequency [175]. Since most osteoporosis studies had small sample sizes, lack of documentation, and limitations for longitudinal studies with high-quality population, it is challenging to identify genetic variants for osteoporosis.

1.9 Summary and research questions

Osteoporosis research has spanned over three decades, yet many aspects of its pathophysiology and genetic influences remain poorly understood. This thesis aims to address critical gaps in our understanding, focusing on the genetic basis of bone mineral density (BMD) and bone loss across diverse populations. While numerous genome-wide association studies (GWAS) have identified loci related to BMD, few studies have explored Asian populations, and almost none have focused on the Vietnamese population.

One key research question is whether bone loss serves as an independent risk factor for fractures, separate from BMD. Previous research suggests that rapid bone loss can increase fracture risk, even in individuals with relatively higher baseline BMD, highlighting the importance of understanding this dynamic relationship. This study contributes to filling these gaps by examining both BMD and bone loss using multiple datasets, each with unique strengths.

Due to the limited availability of repeated BMD measurements in the Vietnamese Osteoporosis Study (VOS), this thesis focuses on a GWAS of BMD rather than bone loss in that cohort (Chapter 6). The VOS analysis represents the first GWAS investigation of BMD in a Vietnamese population, adding valuable genetic insights from an underrepresented

group. In contrast, Chapters 3 and 4 analyze bone loss and its potential role as an independent risk factor for fractures, using longitudinal data from the MrOS and SOF studies. Chapter 5 explores how the COLIA1 gene relates to BMD, bone loss, and fractures in an Australian cohort (the Dubbo Osteoporosis Epidemiology Study), specifically examining whether genetic factors like COLIA1 influence bone loss and fracture risk beyond BMD measures. While pharmacogenomic studies suggest genetic variations in treatment responses, this thesis focuses more on prevention, identifying those at higher risk of bone loss to improve early intervention strategies. This approach aims to enhance fracture prediction models and tailor preventive measures to those at greatest risk.

Rather than focusing on pharmacogenomic evidence for variable treatment effectiveness, such as bisphosphonates, this study centers on prevention, particularly targeting the COLIA1 gene, which has shown consistent associations with BMD and fracture. By exploring its role in bone loss, which requires multiple BMD measurements, the study aims to enhance personalized prevention strategies for those at risk of rapid bone loss and fractures.

This thesis seeks to advance our understanding of the genetic underpinnings of BMD and bone loss, both of which are important for predicting fracture risk. While the GWAS of BMD in VOS is not the first in the world, it represents a significant contribution to genetic research in Vietnam, helping to identify potential genetic markers that could be used for better prediction and prevention strategies in this specific population. By examining the interplay between genetic factors, bone loss, and fracture risk, this research aims to clarify whether targeted genetic insights can enhance current models of fracture prediction and identify those most likely to benefit from specific preventive measures.

1.10 Hypothesis and aims

This thesis hypothesizes that genetic variations, particularly in the COLIA1 gene, influence BMD and bone loss independently, and that bone loss serves as an independent risk factor for fractures, separate from BMD. Additionally, incorporating genetic insights into

prediction models is expected to improve fracture risk assessment and enable more effective prevention strategies.

To address these hypotheses, this study focuses on the following aims:

1. Investigate the role of lifestyle factors in age-related bone loss among elderly men and women using longitudinal data from the MrOS and SOF studies (Chapter 3).
2. Assess the relationship between bone loss, skeletal aging, and mortality in elderly populations (Chapter 4).
3. Explore whether bone loss is an independent risk factor for fractures and examine the impact of COLIA1 genetic variations on BMD, bone loss, and fracture risk in an Australian cohort (DOES) (Chapter 5).
4. Identify novel genetic variants associated with BMD using the Vietnamese Osteoporosis Study (VOS) dataset, contributing to the understanding of genetic influences in an underrepresented population (Chapter 6).
5. Integrate findings and propose future research directions, emphasizing the importance of genetic insights for improving personalized prevention strategies for osteoporosis (Chapter 7).

CHAPTER 2.

STUDY DESIGN AND METHODS

2. Study Design and Methods

2.1 Study design

This study will be combining the cohorts from the Dubbo Osteoporosis Epidemiology Study, the Study of Osteoporotic Fractures, the Osteoporotic Fractures in Men study, and the Vietnam Osteoporosis Study. The study design for each cohort is given below.

2.1.1 The Dubbo Osteoporosis Epidemiology Study (DOES)

2.1.1.1 Dubbo population

Dubbo, a city located approximately 400 kilometers northwest of Sydney, was previously chosen for a study on coronary heart disease and risk factors related to chronic illnesses [176]. The Dubbo Osteoporosis Epidemiology Study (DOES) originated from this earlier investigation in 1989 and has since expanded its scope to include additional health outcomes. Dubbo was selected due to its population's similarity in age and gender distribution to the broader Australian demographic. Furthermore, its relative isolation in terms of medical care ensures that almost all specific health occurrences, such as fractures, are typically observed within the local public hospital or the single private radiological practice.

In 1989, Dubbo's total population was approximately 32,230, primarily Caucasian (98.6%), with 1.4% consisting of indigenous Aboriginal individuals. The age and gender distribution of the population are detailed in Table 2.1. At the study's onset, there were 1581 men, and 2095 women aged 60 years and above residing in Dubbo.

Table 2.1. DOES sample from Dubbo population, baseline and follow-up

Age groups	No of DOES participants	Baseline DOES with BMD[1]	Follow-up DOES (with more than 2 BMD measurement)[2]	Blood sample and BMD[3]
Men				
60 – 64	196	195	166	153
65 – 69	266	265	223	212
70 – 74	181	179	132	133
75 – 79	156	155	109	108
80 +	97	90	46	49
Total	896	884	676	655
Women				
60 – 64	326	326	286	263
65 – 69	337	333	293	278
70 – 74	266	264	226	208
75 – 79	248	239	183	170
80 +	220	202	102	124
Total	1397	1364	1090	1043

[1], Represents the number of subjects who have participated in the study, including those with and without fractures and who agreed to have bone density measured and provided clinical data; [2], represents the number of participants who agreed to have bone density measured and provided clinical data and blood sample; [3],represents the number of participants who agreed to have a second bone density measured and provided follow-up details of clinical data and blood sample.

2.1.1.2 Aims of DOES

The study was originally designed to: (1) determine the incidence and prevalence of all types of fractures in men and women aged 60 years and above residing in Dubbo; (2) identify environmental factors influencing BMD distribution and the risk of osteoporotic fractures among these individuals; (3) analyse bone density changes by conducting repeated BMD measurements; and (4) estimate the burden of osteoporotic fractures on cost, morbidity, and mortality.

2.1.1.3 Study design

DOES was established as a longitudinal, community-based investigation using the Dubbo population as the sampling framework. Through outreach via the electoral roll and media campaigns, all individuals aged 60 years or older as of June 31, 1989, residing in Dubbo and surrounding districts were invited to participate. Subjects were periodically requested to attend follow-up assessments approximately every two years. Participation rates and follow-up statistics are detailed in Table 2.1, showing higher overall involvement among older females (69%) than males (58%). Among participants, about 90% consented to Bone Mineral Density (BMD) measurements, with more than 70% undergoing subsequent BMD assessments.

By 2008, 655 men and 1043 women had provided blood samples and undergone BMD measurements (Table 2.1). Exclusion criteria were applied, excluding participants with specific medical conditions such as Paget’s disease, hyperparathyroidism, Cushing’s syndrome, diabetes mellitus, gastrectomy, malabsorptive syndromes, jejuno-ileal bypass, chronic renal or liver disease, or prolonged immobility. Additionally, individuals recovering from strokes, metastatic malignancies, or those using medications affecting bone metabolism within the past five years were excluded. The study received approval from the Ethics Committee of St Vincent's Hospital, Sydney, and adhered to the National Health Medical Research Council's (NHMRC) Guidelines, following the Declaration of Helsinki, 1964. Written informed consent was obtained from all participants.

2.1.1.4 Data collection

Data collection was conducted through direct interviews conducted by a nurse coordinator at the study's commencement and subsequent visits. Each participant completed a structured questionnaire to gather information on anthropometric characteristics, general health, lifestyle factors, and fractures. Bone mineral density was measured during each visit whenever possible. Blood samples were collected and stored at -80°C.

2.1.1.4.1 Anthropometric characteristics

The following anthropometric data were collected, including age, height, and weight. Date of birth (DOB) was acquired from a questionnaire during the study's entry, and participants' age (in years) was calculated from DOB to the date of their first visit. Height and weight were measured at the initial entry. Height, taken without shoes, was measured using a wall-mounted stadiometer with precision to the nearest 0.1cm. Weight, without shoes and in light clothing, was measured with an electronic scale, recorded to the nearest 0.1kg. Body mass index (BMI) was calculated as the ratio of weight (in kg) divided by the square of height (in meters).

2.1.1.4.2 Clinical history

A structured questionnaire was used to collect details on overall health, specifically focusing on reproductive histories such as age at menarche and menopause, the number of live births (parity), the number of pregnancies (gravidity), occurrences of falls in the past 12 months, and any familial history of osteoporosis or fractures.

2.1.1.4.3 Bone mineral density

Bone mineral density (BMD, g/cm^2) was measured at the femoral neck (FNBMD) and lumbar spine L1-L4 (LSBMD) using dual-energy X-ray absorptiometry (DXA) with a DPX densitometer (GE-LUNAR Corp, Madison, WI) during initial and subsequent visits. Measurements at the femoral neck were typically taken on the right side unless a hip replacement or fracture had occurred, in which case the left side was scanned. The radiation dose for this method is less than 0.1 μGy. The coefficient of variation (CV) for BMD

measurements within the same clinic (intra-clinic) is approximately 1.5% for the lumbar spine and 1.3% for the femoral neck, ensuring high precision and consistency in measurements across repeated assessments [91].

2.1.1.4.4 Genotyping

Blood samples from each participant were collected at baseline and in each subsequent visit for extracted leukocytes. DNA was extracted from leukocytes with proteinase K and phenol chloroform for PCR and genetic analyses [136]. The G to T's polymorphism in the first 265-bp region of intron 1 of the *COLIA1* gene was amplified with MscI (New England Biolabs, Beverly, MA) and digested with an isoschizomer of BalI. Then, PCR products were analyzed by 3% agarose gel electrophoresis. The restriction site of *COLIA1* genotypes were referred to as *GG, GT,* and *TT*, with *T* being the minor allele. Thirty blood samples were randomly selected and re-genotyped to confirm the genotyping accuracy with 100% consistency. The process of DNA extraction and PCR of *COLIA1* gene were performed by colleague Dr. Bich Tran [177].

2.1.1.4.5 Ascertainment of fractures

All incident fractures that occurred during the study were identified among Dubbo residents through radiology reports from the only two centres providing X-ray services in the area. Fractures were verified based on radiology reports and ascertained during follow-up visits through personal interviews conducted near the time of occurrence. Only fractures caused by minimal trauma, such as a fall from a standing height or lower, were included in the analysis [99, 178], in keeping with the standard definition of fragility fractures associated with osteoporosis. High-impact trauma fractures (e.g., from motor vehicle accidents) and pathological fractures related to malignancy or non-osteoporotic bone disease were excluded. Although previous research has demonstrated that BMD is strongly associated with all fractures-including those from more than minimal trauma-the present study aimed to isolate osteoporotic fractures where bone fragility is the primary cause [179]. Excluding traumatic fractures reduces heterogeneity in etiology and allows for clearer identification of BMD-related risks in aging populations. Therefore, fractures due to high-impact trauma (e.g., motor

vehicle accidents) or pathological conditions such as cancer or non-osteoporotic bone diseases were excluded. Additionally, fractures of the skull, fingers, toes, and cervical spine were not considered.

Included fractures were categorized as fragility fractures, with specific subgroups for hip fractures occurring at the femoral neck and vertebral fractures occurring at the lumbar spine. Vertebral fractures were considered only if there was recent clinical evidence or a radiograph indicating a fracture within the last two years. Asymptomatic vertebral fractures were not assessed. The mortality status of participants was determined through lists of local funeral directors, newspapers reports, and verified using death certificates from the New South Wales Registry of Births, Deaths and Marriages [180].

2.1.2 Study of Osteoporotic Fractures (SOF)

2.1.2.1 Aims of the study

The SOF study was a prospective observational study that commenced in 1986, involving a cohort of women aged 65 or older in the United States. Its objectives were to: (1) record information based on different physical and mental evaluations and follow-up assessments every four months to help identify the risk factors of osteoporosis in aging women.; (2) gather data on risk factors associated with osteoarthritis (OA); and (3) analyse baseline hip and hand films to evaluate the presence and severity of OA.

2.1.2.2 Study design

The SOF was a longitudinal, multi-center study designed to evaluate the risk factors associated with osteoporotic fractures in women aged 65 and older. Active from 1986 to 2017, it initially enrolled 10,366 women in four U.S. cities: Baltimore, MD; Minneapolis, MN; Pittsburgh, PA; and Portland, OR. Initially, the cohort consisted of 9,704 Caucasian women, identified as the group at highest risk for osteoporotic fractures. Later, between 1997 and 1998, an additional 662 African American women were included to enhance diversity. Women with bilateral hip prostheses or those unable to walk unassisted were excluded at the start. Throughout the study, participants attended regular clinical visits for comprehensive

physical and mental evaluations. Additionally, they were contacted every four months via phone or mail for follow-up assessments to gather data on falls, fractures, and vital status.

The study adhered to the Declaration of Helsinki guidelines. Approval for the study (ID: GO 21/1164) was granted by the local ethics committee. Participants were provided with comprehensive information, both verbally and in written form, regarding the study details, and their consent was acquired.

2.1.2.3 Data collection

Data collection is conducted at the four clinical centers following standardized protocols and comprehensive operation manuals. To ensure proficiency and consistency in the performance of measurement protocols, clinical staff undergo centralized training by the coordinating center at the beginning of each examination cycle and receive subsequent re-certification during annual site visits.

Information from the SOF is managed using a distributed data system. Initially gathered through interactive data entry with local editing, it is then forwarded to the San Francisco Coordinating Center for further refinement and the creation of an SAS dataset. Accessible data can be downloaded from the study's website (sofonline.ucsf.edu). Mortality ascertainment involved monitoring participant deaths through death certificates and a review of hospital discharge summaries.

2.1.2.4 Bone mineral density

BMD at the lumbar spine and femoral neck was first measured during phase 2 (1989-1990), which is considered the baseline for this analysis. DXA scans were conducted using Hologic QDR-1000 scanners (Hologic Inc., Waltham, MA). Of the 9,704 subjects originally enrolled, 87% of surviving women returned for phase 2, and 7,659 women had a complete set of BMD measurements. To ensure consistency across different clinical sites, phantom calibration was conducted at the start of the study and repeated three years later to assess interscanner variability. The in vivo interscanner CV was 1.52% for spine measurements (three clinics) and 1.20% for femoral neck measurements (four clinics), ensuring consistent

measurement precision across sites [181]. All DXA data were electronically transferred to a centralized coordinating center for review and storage. Quality control procedures included random checks of data entry accuracy, review of flagged cases with results outside 4 standard deviations (SD) of the mean, and reanalysis as needed. Of the women who participated in the study, 4124 underwent repeat BMD measurements using the same equipment whenever possible, providing a reliable basis for longitudinal analysis.

2.1.2.5 Lifestyle factors

Participants' cigarette smoking was categorized into three groups—current, former, and never smokers—based on their responses at the time of the final outcome measurement during the initial 10-year follow-up period. This classification relied on a single question. Women who had smoked fewer than 100 cigarettes in their lifetime were categorized as never smokers.

Alcohol consumption was recorded as the number of standard drinks per day, defined as a 12-ounce (360 mL) can of beer, a 5-ounce (150 mL) glass of wine, or a mixed drink containing roughly 1.25 ounces (38 mL) of liquor. At baseline, alcohol consumption was categorized into two groups: current drinkers and non-drinkers. Current drinkers were identified as women who reported having at least one drink per month either before the baseline or follow-up visit, while never users indicated no use of alcohol at any time. The analysis excluded heavy drinkers, defined as women consuming 14 drinks per week or more (n=411 at baseline), as the study focused on moderate alcohol use.

The Block Food Frequency Questionnaire (FFQ)[182], which included queries on alcohol consumption, as well as vitamin and mineral supplementation (including calcium), was completed before the clinical visit. Responses from the FFQ were utilized to calculate nutritional summary variables based on the Block Dietary Data Systems (Berkeley, California, USA)[183]. During visit 4 (1992-1994), weekly calcium intake was measured in milligrams per week (mg/week) through a comprehensive 21-item dietary questionnaire developed by Block Dietary Systems. Protein intake was initially measured in grams per day

(g/day) during visit 6 (1997-1998) and converted to milligrams per week (mg/week) for consistency with calcium intake.

Physical activity was evaluated using a modified Paffenbarger survey [184], which involved inquiries about engagement in different sports, recreational activities, and walking. The summary measure utilized for this analysis was the average weekly caloric expenditure over the 12 months prior to the baseline examination.

2.1.3 Osteoporotic Fractures in Men (MrOS)

2.1.3.1 Aims of the study

The Osteoporotic Fractures in Men (MrOS) is a prospective cohort study focusing on investigating risk factors associated with vertebral and non-vertebral fractures in older men, while also examining the consequences of these fractures. The aims of this study were: (1) to establish the skeletal factors determining fracture risk in older men, (2) to identify lifestyle and medical factors associated with fracture risk, (3) to determine the contribution of fall frequency to fracture risk in older men, (4) to assess the impact of androgen and estrogen concentrations on fracture risk, (5) to explore the impact of fractures on quality of life, (6) to identify gender-based variations in both predictors and outcomes of fractures, (7) to collect and store serum, urine, and DNA for future analyses based on emerging evidence in aging and skeletal health, and (8) to establish the correlation between bone mass/fracture risk and prostate diseases.

2.1.3.2 Study design

The MrOS study includes men aged 65 years or older residing in the community who are ambulatory. The inclusion criteria were designed to ensure a diverse representation of older men in the study cohort. A total of 5,994 men aged 65 years and older volunteered from six clinical centers across the United States (Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Pittsburgh, PA; Portland, OR; and San Diego, CA). Recruitment commenced in 2000 and followed up for 15 years. The study design was described in a previous study [185, 186]. Participants were primarily recruited through mailed invitations to men living in the

communities surrounding the clinical sites. Additionally, certain sites supplemented recruitment efforts through advertisements in community and senior newspapers, as well as presentations to community groups.

2.1.3.3 Data collection

Lifestyle factors in the MrOS study were assessed through a combination of interviewer-administered and self-administered questionnaires, as well as direct measurements. At baseline (visit 1), height and weight were measured, and body mass index (BMI) was calculated as weight in kilograms divided by height in meters squared. Participants also provided details about their education level, marital status, medical history, medication use, dietary habits, physical activity, smoking, and alcohol consumption through a self-administered questionnaire. During the baseline visit, participants brought their prescription medications to the clinic, where study staff recorded the names and doses of all medications. Throughout the study, participants' status (alive or deceased) and causes of death were confirmed by examining state-issued death certificates.

2.1.3.4 Bone mineral density

BMD (in g/cm^2) at the total body, lumbar spine, and proximal femur were measured by dual energy X-ray absorptiometry (DXA, QDR 4500, Hologic, Inc, Waltham, MA, USA) at baseline; with follow-up measurements taken at the second clinic visit. However, due to resource limitations and participant availability constraints, assessments of radial BMD were not performed. Each U.S. clinical site utilized DXA machines from the same manufacturer and model (QDR 4500, Hologic, Inc, Waltham, MA, USA). To ensure precise and optimize longitudinal measurements across at the six clinical sites, quality assurance measures were integrated into the DXA protocols. In addition, all DXA technicians underwent centralized training before the study measurements commenced. Furthermore, machine performances were monitored daily by scanning phantoms at each clinical site.

The intra-clinic coefficients of variation for spine phantoms ranged from 0.34% to 0.42%, while for hip phantoms, ranged from 0.37% to 0.58%, were considered within acceptable limits. The inter-clinic CVs were 0.6% for the spine and 0.9% for hip, and the

maximum difference between means recorded at 1.4% for spine and 2.2% for the hip. To adjust for potential inter-clinic variations, statistical models included indicator variables for the different DXA scanners.

2.1.3.5 Lifestyle factors

Smoking status was self-reported at visit 1 and categorized as non-smoker, ex-smoker, or smoker, with data collected only at this visit. Alcohol intake was assessed via interviewer-administered questionnaires and quantified based on the number of drinks consumed per week, categorized into six groups: 0 drinks/week, <1 drink/week, ≥1 to <7 drinks/week, ≥7 to <14 drinks/week, ≥14 to <21 drinks/week, and ≥21 drinks/week.

Physical activity levels were assessed at visit 3 using the Physical Activity Scale for the Elderly (PASE) and calculated as total kilocalories burned per week based on reported average energy expenditure [187]. Although physical activity data were collected at both visit 3 (n=3,354) and visit 4 (n=1,516), only visit 3 data were analyzed to ensure a more representative sample of the population and to reduce confounding.

Dietary calcium intake and dietary protein intake were assessed using a version of the Block Food Frequency Questionnaire (FFQ) tailored for men. The FFQ included 69 food items and additional questions on food preparation and low-fat options. Dietary calcium intake was calculated as the sum of daily calcium intake (mg) and daily calcium from supplements (mg). Dietary protein intake was further categorized into daily animal protein intake (g) and daily vegetable protein intake (g). Dietary intake data were collected at visit 1.

2.1.4 The Vietnam Osteoporosis Study (VOS)

2.1.4.1 Aims of the study

This study aimed (1) to develop a Vietnamese genetic database and identify genes associated with increased susceptibility to chronic non-communicable diseases, including diabetes, hypertension, obesity, osteoporosis, and joint degeneration (2) to create risk assessment tools to identify individuals with high-risk genes, improving prevention, early

diagnosis, and treatment selection for non-communicable diseases (3) to establish a foundation for future research to enhance community health and disease prevention.

2.1.4.2 Study design

This study was part of the Vietnam Osteoporosis Study (VOS), a population-based, family, long-term, prospective study. The inclusion criteria were men and women aged 18 years and older from Ho Chi Minh City and nearby rural areas. Recruitment methods included community outreach, where the study team collaborated with community organizations to compile a list of potential participants, and a random selection process using a computer program to identify individuals meeting age and sex criteria. These individuals, along with their family members, were invited to participate. Additionally, participants were recruited through television, the Internet, and flyers distributed at universities. The flyers, written in Vietnamese, outlined the study's objectives, procedures, and participant benefits. All participants attended the Bone and Muscle Research Laboratory at Ton Duc Thang University for clinical assessments, where they received free health check-ups and lipid analyses, but no direct financial compensation was provided [188]. The protocol and procedures of this study were approved by the Medical Ethics Committees of the People's Hospital 115 and the Pham Ngoc Thach University of Medicine on August 6, 2015 (approval number: 297/BV-NCKH). All participants provided written informed consent.

2.1.4.3 Data collection

Information on participants' demographic factors, clinical history, medication use, lifestyle factors, physical activity, dietary habits, history of falls and fractures, and anthropometric factors were gathered using a structured questionnaire administered by a trained interviewer. Height and weight were measured using an electronic portable, wall-mounted stadiometer (Seca Model 769; Seca Corp, CA, USA) without shoes or hats or heavy clothing. Body mass index (BMI) was calculated by dividing the weight in kilograms by the square of the height in meters. Participants were also asked to provide information on current and past smoking habits, as well as their alcohol consumption, both at present and over the past 5 years.

2.1.4.4 Bone mineral density

The measurements of areal BMD at the lumbar spine, femoral neck, and total hip were obtained using dual-energy X-ray absorptiometry (Hologic QDR 4500, Hologic Corp, Bedford, MA, USA) for this study. Three BMD phenotypes were distinguished and analyzed: lumbar spine (L2-L4) (LSBMD), femur neck (FNBMD), and total hip (HTOTBMD). These measurements were standardized using a phantom before each scan to ensure consistency and were performed by a qualified radiology technologist. The coefficient of variation for BMD measurements in this study was 1.5% for the lumbar spine and 1.7% for the hip, reflecting the intra-clinic precision of the measurement [189].

2.1.5 Study cohort differences and implications

This study utilizes data from four different osteoporosis cohorts: the Dubbo Osteoporosis Epidemiology Study (DOES; Australia), the Study of Osteoporotic Fractures (SOF; USA), the Osteoporotic Fractures in Men (MrOS; USA), and the Vietnam Osteoporosis Study (VOS; Vietnam). While these cohorts offer important insights into osteoporosis risk factors and bone health, we must recognize the differences in study design, recruitment approaches, participant demographics, and data collection methods, as these variations may impact study results and the generalizability of findings.

The SOF and MrOS cohorts, described in Chapters 3 and 4, were designed as separate studies for women and men, respectively, and were conducted across multiple clinical centers in the United States. Although these cohorts focus exclusively on one sex each, analyses in Chapters 3 and 4 were conducted separately for men (MrOS) and women (SOF). Chapter 3 examines the associations between lifestyle factors and bone loss, while Chapter 4 investigates the relationship between bone loss and mortality. In contrast, the DOES cohort, outlined in Chapter 5, is a community-based longitudinal study in Dubbo, Australia, that includes both men and women aged 60 years and older. The VOS cohort, described in Chapter 6, differs significantly as it is a population-based study conducted in Ho Chi Minh City, Vietnam, that includes both men and women aged 18 years and older. Additionally,

VOS employed diverse recruitment methods, including community outreach, random selection, and family-based enrollment, distinguishing it from the other cohorts.

There are several differences across these cohorts that should be considered when interpreting the results. Firstly, geographical and ethnic variations are notable, as participants in DOES, SOF, and MrOS are predominantly Caucasian, whereas those in VOS are Vietnamese. These ethnic differences may influence genetic predispositions, environmental factors, and lifestyle behaviors related to osteoporosis risk. Secondly, differences in recruitment strategies across the studies may have led to variations in participant characteristics. Thirdly, data collection methods were not uniform across the cohorts, as BMD measurements were conducted using different DXA machines, which could introduce variations in precision despite calibration efforts. Additionally, in SOF and MrOS, lifestyle data were collected through self-administered questionnaires, which may have introduced recall bias or self-reporting inaccuracies.

Given these differences, each cohort is analyzed separately in this thesis, with no direct statistical comparisons between them. However, variations in study design, recruitment strategies, and data collection methodologies may influence the interpretation of findings. Chapter 7 will further examine these limitations and their implications for osteoporosis research

2.2 Genome-wide association study

Genome-wide genotyping of about 1 million single-nucleotide polymorphism (SNP) will be performed in more than 4000 participants in the Dubbo Osteoporosis Epidemiology Study by Illumina chip. Previous known BMD loci will be tested for replication in the 23andMe cohort which is a training dataset [147].

2.3 Data analysis

2.3.1 Analysis of fracture risk

Cox proportional hazard model was used to identify potential indicators for fractures [190].In this model, the time to fracture is the dependent variable and is represented as an exponential function involving various risk factors, as follows:

$$h_{i(t;x_1;x_2,\ldots,x_p)} = h_0(t)e^{\sum_{j=1}^{p}\beta_j x_{ji}}$$

Here, h_i represents the hazard or risk function associated with the i-th individual at various time points (t = 1, 2, 3,...,T). The term $h_0(t)$indicates the baseline hazard (when all x's =0). The variable x_{ji} signifies the value of the *j*-th independent variable for the i-th individual, while β_j represents the regression coefficient linked to that specific variable. These independent variables include factors like lifestyle, age, bone mineral density, and body mass index

The analyses started by identifying a combination of variables with the highest ability to differentiate between fracture and non-fracture cases. This was accomplished through the application of three algorithms: stepwise, forward, and backward elimination. The construction of the "final" model relied on the alignment and coherence of these algorithms.

The parameter estimates of Cox proportional hazard model were evaluated for significance using the likelihood ratio statistic [191]. A conventional p-value threshold of < 0.05 was used to determine statistical significance throughout the analysis. This level balances the risk of Type I and Type II errors and is consistent with standard practice in epidemiological studies. Variable selection for the final multivariable models was informed by both stepwise selection and clinical plausibility, acknowledging that this exploratory approach has limitations compared to methods based on causal inference frameworks. While p-value thresholds may vary depending on study size and statistical power, the use of < 0.05 in this study reflects conventional practice. Further details regarding the p-value thresholds

and the impact of study size on significance testing will be elaborated in subsequent chapters, which discuss the different studies conducted.

The assumption of proportional hazards across various levels of risk factors was assessed by examining the linearity of log (log $(-\log(S(t_i)_j))$) plots, where $S(t_i)_j$ represents the *j*th survival time for the ith level (i = 1, 2) of each risk factor. To test whether the predictions made by the proportional hazard model hold true for a different set of subjects, the data were randomly split into two nearly equal samples. Using the estimated coefficients [192], both the fracture risk score and the concordance index were calculated, representing the area under the receiver operating characteristic (ROC) curve [193].

Although modern causal inference frameworks, such as directed acyclic graphs (DAGs), offer more robust tools for confounder identification and model building, we employed stepwise variable selection as a pragmatic exploratory method. This decision was made due to the lack of pre-specified causal structure and the primarily observational nature of our data. The stepwise process allowed us to identify variables associated with fracture risk within the available dataset, while acknowledging its limitations—including potential overfitting and the risk of identifying statistically significant but clinically trivial associations. These trade-offs are discussed further in the limitations section.

2.3.2 Analysis of the rate of bone change

The rate of bone change in BMD was analyzed using mixed-effects linear regression model to capture both the population-level effects and individual-specific variations in BMD over time. This approach accounts for repeated measurements of BMD within individuals, allowing for the estimation of both fixed effects (the average rate of bone change across the population) and random effects (the differences in bone change patterns between individuals).

Here, BMD_{it} represents the BMD of the i-th subject (i = 1, 2, ..., n) at the t-th visit (t = 0, 1, 2, ..., m). The time since baseline is denoted as T_{it} , where T_{i1} = 0) indicates the

baseline visit. The following mixed-effects model was used to estimate the rate of bone change:

$$BMD_{\mathrm{it}} = \alpha + \beta T_{\mathrm{it}} + r_{\mathrm{i}} + s_{\mathrm{i}}\mathrm{T}_{\mathrm{it}} + \mathrm{e}_{\mathrm{it}} \quad [2.3.1]$$

In the formula above, α represents the population mean baseline BMD, normalized to a population SD, allowing for comparisons across individual. β stands for the fixed effect for time (T_{it}), representing the average rate of BMD change over time (g/cm^2/year) across the population. r_{i} represents the random intercept for the i-th subject, capturing the individual-specific deviation from the population mean baseline BMD, while s_{i} represents the random slope for the i-th subject, capturing the individual-specific deviation in the rate of BMD change. The term e_{it} represents the residual error term, which includes measurement error and within subject variation.

In terms of BMD normalization and machine variability, BMD values are normalized to the population SD to allow for standardized comparisons across individuals. However, absolute BMD values can vary between different densitometry machines due to differences in calibration. While normalization mitigates much of this variation, machine-based differences may still affect the precise measurement of absolute BMD values. The mixed-effects model accounts for individual-specific deviations (random effects), but the rates of change in BMD are based on normalized values rather than the absolute values from each machine.

Despite normalization, some machine-specific variability may persist. While this analysis assumes that normalization to SD handles much of the variability, additional random effects or covariates could be included in future analyses to explicitly model machine-specific variability.

In terms of estimation of variance and rate of change, the standard deviation of the residuals is estimated using the root mean square of ε_{ij}, which captures the random variation within subjects and can be considered the estimation error.

$$\sigma_i = \sqrt{\frac{1}{m-2}\sum_{j=1}^{m}(\varepsilon_{ij})^2}$$

[2.3.2]

When *m* represents the count of measurements for the *i*-th participant. The intrasubject variance of the slope for any *i*-th subject, σ_{bi}^2, can be expressed as:

$$\sigma_{bi}^2 = \frac{12m\,\sigma_i^2}{t^2(m+1)(m+2)}$$

[2.3.3]

This applies to *m* + 1 equally spaced measurements conducted over *t* years. Determining the standard error of the rate of change for an individual is done by estimating σ_i^2.

In terms of group comparison and hypothesis testing, in a model of linear bone loss among a group of *n* subjects, the rates of bone change can be summarized by the mean and variance of the individual slopes b_i (representing linear rates of bone change):

$$\bar{b} = \frac{1}{n}\sum_{i=1}^{N} b_i$$

and

$$\sigma_{\bar{b}}^2 = \frac{1}{N-1}\sum_{i=1}^{N}\left(b_i - \bar{b}\right)^2$$

[2.3.4]

The variance of the mean rate of bone change, $\sigma_{\bar{b}}^2$, encompasses both individual slope variance and the between-subject variance in rates of bone change:

$$\sigma_{\bar{b}}^2 = \frac{1}{n}\left[\sigma_b^2 + \frac{12m\,\sigma_i^2}{t^2(m+1)(m+2)}\right]$$

When testing for a difference of Δ mg/cm2/year between two groups regarding the rate of bone change, with significance levels α and a type II error of β (power = 1 - β), the required sample size N per group depends on the difference in rates of change between the groups, as well as intersubject and intrasubject variations. These calculations follow closely with those presented by Schlesselman (1973), assuming constant intrasubject variance $\sigma_i^2 = \sigma^2$.

The required sample size N is calculated as follows:

$$N = \frac{2\left(\mathrm{Z}_\alpha + \mathrm{Z}_\beta\right)^2}{\Delta^2} \times \left[\sigma_b^2 + \frac{12m\,\sigma_i^2}{t^2(m+1)(m+2)}\right]$$

[2.3.5]

In the formula above, σ_b^2 represents the between-subject variance in the rate of bone change, while σ_i^2 stands for the within-subject variance, including measurement error. m represents the number of measurements per subject, and t is the total follow-up time.

Since BMD values are normalized to a population SD, the variances σ_b^2 and σ_i^2 reflect individual variability in normalized BMD rather than differences caused by measurement devices. Machine-specific variability was accounted for through

centralized cross-calibration conducted by study investigators prior to public data release. Therefore, normalized BMD values used in this analysis reflect biological differences between individuals and are not influenced by scanner differences.

BMD change was modelled as a function of time since enrolment to maintain consistency in follow-up intervals. However, we recognize that modeling BMD by age may better reflect physiological processes and allow comparison with prior studies. Future models may incorporate age-based modeling to validate findings.

2.3.3 Analysis of GWAS data

For GWAS, common SNPs with a minor allele frequency threshold of more than 1% will be imputed to ensure accurate ascertainment. Mix-effects logistic regression adjusted for age, sex and weight will be used to test additive (per allele) genetic effects. Hardy-Weinberg equilibrium law will be performed to check the frequencies of alleles and genotypes of all genotyped variants. All unbiased estimates of effect size for each SNP will be assessed at a genome wide significance level ($P<10^{-8}$). Linkage disequilibrium score regression will be conducted to estimate the degree of shared genetic risk factors between bone loss, BMD, and fracture.

CHAPTER 3.

ASSOCIATION BETWEEN LIFESTYLE FACTORS AND AGE-RELATED BONE LOSS

3. Association between Lifestyle Factors and Age-related Bone Loss

3.1 Introduction

Osteoporosis is a chronic condition characterized by reduced bone mineral density (BMD) and deterioration of bone microarchitecture, leading to increased bone fragility and a heightened risk of fractures. Despite decades of research, understanding how lifestyle factors interact with genetic predispositions to influence bone loss remains limited. This study uniquely examines the combined effects of multiple lifestyle factors on bone loss, using large, well-characterized cohorts, filling a critical gap in existing literature. Bone loss is a critical component not only in increasing fracture risk but also in contributing to increased mortality among the older [194-196]. Evidence from an Australian cohort also supports this relationship, demonstrating that concurrent declines in muscle mass and bone density are associated with increased mortality in older adults [197]. Therefore, interventions targeting bone loss, such as anti-fracture pharmacological treatments, can reduce both fracture risk and mortality. Additionally, modifiable lifestyle factors—including smoking cessation, physical activity, and nutritional intake—have been linked to bone health, making them potential targets for population-based interventions to mitigate bone loss and promote healthy aging.

Bone loss typically accelerates with advancing age, particularly in individuals over 60 years old, but there is significant variability between individuals [198]. While some experience rapid bone loss, others show a more gradual decline. Those with accelerated BMD loss are at a heightened risk of fractures [198-201] and face increased mortality compared to individuals who maintain stable BMD, in both men [202] and women [203]. Thus, understanding the dynamics of bone loss is essential to identify individuals at greatest risk of adverse outcomes, such as fractures and mortality.

Although genetic predisposition plays a role in osteoporosis, modifiable lifestyle factors remain significant, lifelong determinants of bone health. For instance, cigarette smoking is consistently associated with decreased BMD [204], while alcohol consumption shows a complex relationship with bone health: excessive intake raises fracture risk [205],

but moderate consumption may slow BMD loss [206]. Physical activity is strongly associated with higher BMD [207-209], while inactivity contributes to lower BMD and higher fracture risk [210]. Similarly, inadequate calcium intake leads to a negative calcium balance and contributes to bone loss [211]. The impact of dietary protein on BMD remains debated, with mixed findings from cross-sectional and short-term studies [212-215].

Despite extensive research on individual lifestyle factors and BMD, few studies have simultaneously examined how multiple lifestyle behaviours—such as smoking, alcohol consumption, physical activity, dietary calcium, and protein intake—interact to influence longitudinal bone loss. This study aims to address that gap by investigating how these modifiable factors, alongside environmental influences such as comorbidities and nutritional status, contribute to bone loss in older men and women. Using data from the Study of Osteoporotic Fractures (SOF) for women and the Osteoporotic Fractures in Men (MrOS) for men, we hypothesize that lifestyle factors—including cigarette smoking, alcohol consumption, dietary calcium and protein intake, and physical activity—are associated with bone loss. We test this hypothesis by examining the relationships between these lifestyle choices and bone loss, contributing to a comprehensive understanding of how lifestyle modifications may influence the risk of osteoporosis and fragility fractures.

3.2 Study design and methods

The current chapter utilized data from the Study of Osteoporotic Fractures (SOF) for women and the Osteoporotic Fractures in Men (MrOS) for men. Detailed study design and methods are provided in Chapter 2. In SOF, relevant data were collected at Visit 6 (1997-1998), while in MrOS, baseline data were collected at Visit 1 (2000-2002), aligning the time points used for lifestyle factor measurement.

To ensure reliable longitudinal estimates of bone loss, only participants with at least three BMD measurements over time were included (women = 5,343; men 3,577). Participants with missing data on drinking status (n=3 MrOS), smoking status (n=18 SOF and 1 MrOS), and physical activity level (n=31 SOF) were excluded, resulting in a final sample of 5,292 women and 3,557 men (Figure 3.1). The primary aim of the statistical

analysis was to explore the association between lifestyle factors and rate of bone change in both men and women. The analysis began by examining dietary calcium intake, dietary protein intake, and physical activity level.

In the SOF cohort, dietary calcium intake was calculated as the sum of daily calcium (mg/day) and average daily calcium from supplement (mg/day). Dietary protein intake (g/day) was only collected at visit 6. In MrOS, dietary calcium intake included both daily calcium intake (mg) and calcium from supplement (mg). Dietary protein intake was categorized into daily animal and vegetable protein intake (g). Data were collected at visit 1 for MrOS and at visit 6 for SOF, as these visits provided consistent measurements for comparison between the two studies. Physical activity levels were self-reported for women, based on total kilocalories burned per week from activities and walking over the past year. For men, physical activity levels were calculated by kilocalories burned per week based on reported average measured energy expenditure.

Given the highly skewed distributions of dietary calcium intake, dietary protein intake, and physical activity levels, these variables were categorized into quartiles before analysis. Quartile cut points were derived from the sex-specific distributions within each cohort. Alcohol intake was assessed differently for men and women. In men, alcohol consumption was assessed by interviewer-administered questionnaires and categorized based on drinks consumed per week (0 drink/week, < 1 drink/week, ≥1 - <7 drink/week, ≥7 - <14 drink/week, ≥ 14 - < 21 drink/week, and ≥21 drink/week). For women, alcohol consumption was self-report based on the number of drinks consumed per week in the past 30 days. To maintain consistency, alcohol consumption in women was categorized into the same six groups as used for men.

For the fully adjusted models, we selected variables based on a literature review: age, race/ethnicity (only in MrOS), BMI, osteoporosis medication use, and concomitant diseases. In MrOS, race/ethnicity was self-identified as White, African American, Asian, and Other. This variable was excluded from SOF as the initial cohort (visit 1) was exclusively Caucasian, and African American participants were added only after visit 6. Due to the difference in recruitment timing, only Caucasian participants were included for comparison.

Participants were considered to be using osteoporosis-related medications if they self-reported the use of any of the following during follow-up: fluoride, calcitonin, bisphosphates (alendronate, etidronate), thyroid hormone, or estrogen. Concomitant diseases were defined as self-reported cardiovascular diseases (CVD), all types of cancer, or osteoporosis. Although osteoporosis is the primary outcome of interest, we included it among comorbidities to account for pre-existing diagnoses that may influence BMD change due to earlier disease management or medication use.

Given that CVs tend to increase as BMD decreases, potentially introducing more variability in measurements at lower BMD levels, our analysis addressed this issue using a mixed-effects model that estimates both baseline and change simultaneously by incorporating random effects, which allow individual differences in BMD trajectories to be modelled without including baseline BMD as a separate covariate. This approach helps to estimate the rate of bone loss more accurately over time. Mixed-effects models are ideal for longitudinal data with repeated measurements, as they account for individual differences in both baseline BMD and changes over time [216-218]. By adjusting for baseline BMD, the model helps to mitigate the increased variability that can occur at lower BMD values, providing more precise estimates of bone loss rates. Additionally, the model's use of random effects for each individual accommodates differences in starting points and rates of BMD change, offering a robust analysis across a range of BMD values.

Total calcium intake, dietary protein intake, and physical activity level had highly skewed distributions, so they were categorized into quartiles based on the present data distributions before analysis. All participants reporting implausible estimated total energy intake, either extremely low (< 500 kcal/day) or extremely high (> 5000 kcal/day), and those who had extremely high dietary protein (>1.0 g/kg/d) were excluded. This threshold was selected based on prior studies in older populations to reduce potential misreporting or outlier effects [219-221]. After verifying the requirement of having at least three BMD measurements, the missing data for age (3 SOF), height (6 MrOS), and BMI (6 MrOS) were replaced with the mean value of the respective variable to maximize data utilization. A linear mixed-effects regression was used to estimate the rate of change in BMD for each

participant. The advantage of this model is that it accounts for repeated measures, missing data, and both between- and within-subject variation [216-218]. BMD change was modelled as a function of time for each individual; with the annual rate of bone change (%/year) calculated by expressing the individual slope as a percentage of their initial BMD value to standardize across baseline variation, multiplied by 100. The collection of slopes across individuals was modelled as a linear function of lifestyle factors: smoking status, drinking status, total calcium, dietary protein intakes, and physical activity level.

The associations between lifestyle factors, treated as categorical variables, and bone loss were analyzed using the Kruskal-Wallis test to evaluate overall differences across categories. Given that the Kruskal-Wallis test is an omnibus test, pairwise comparisons were conducted only when the overall test was significant, using Dunn's test with Bonferroni correction to control for multiple comparisons. Additionally, a relative importance analysis using Gromping's LMG method [222] differentiated the relative importance of correlated regression variables in multivariate linear models, quantifying each factor's contribution to the overall model. A P-value < 0.005 was considered statistically significant to minimize false positives [223]. All analyses were performed with R [224].

However, we did not incorporate time-varying exposures in this analysis due to inconsistent timing and availability across cohorts. Using baseline values ensured comparability between SOF and MrOS and minimized bias from differential follow-up durations. Sample sizes differed slightly between men and women due to differences in initial cohort sizes and availability of complete lifestyle and BMD data. Additional exclusions for missing lifestyle factors and extreme dietary reports further reduced the analytical sample in subgroup analyses.

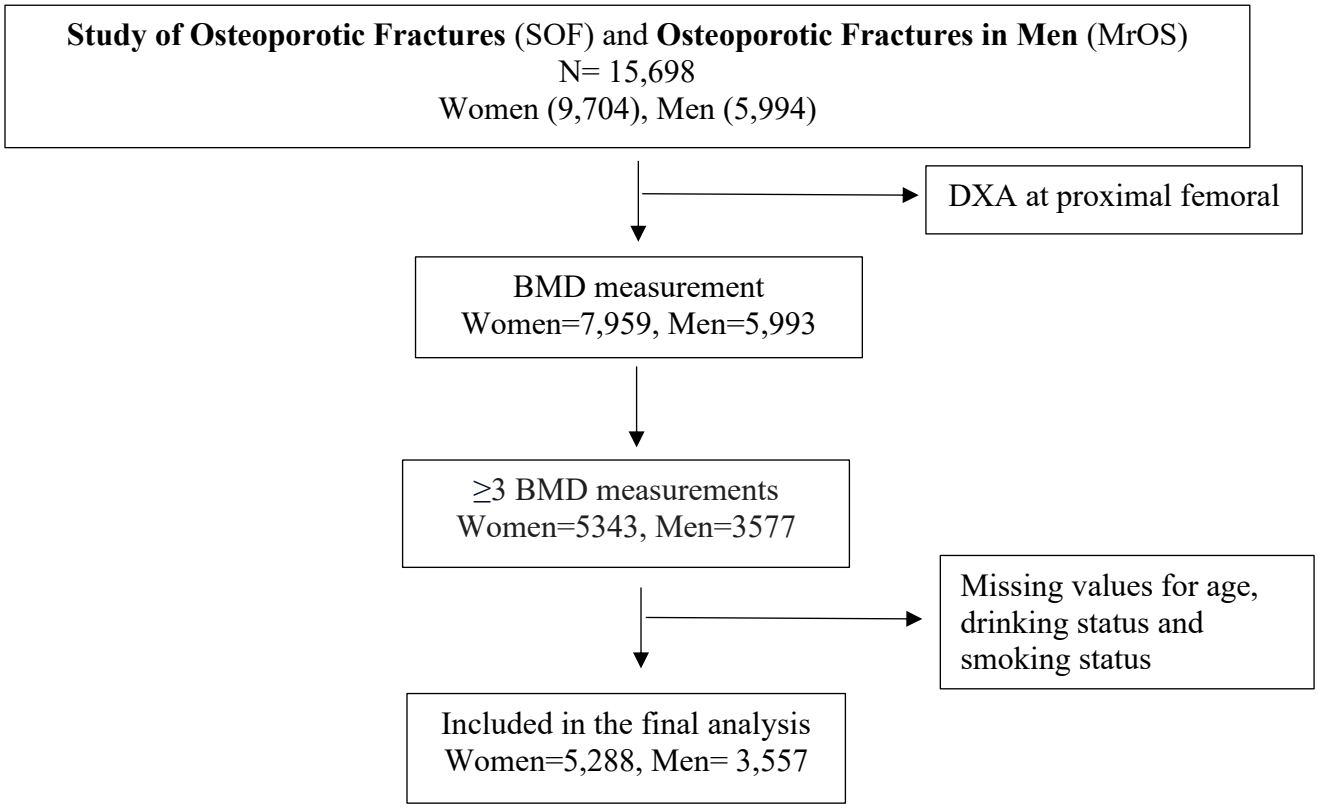

Figure 3.1 Flow chart of SOF and MrOS participants according to the analytical samples. Abbreviations: BMD, bone mineral density; M, men; W, women.

3.3 Results

3.3.1 Baseline characteristics

The analysis included 5,288 women and 3,557 men with ≥3 BMD measurements over a median follow-up of 7.8 years (IQR: 3.5-12.2) for women and 6.6 years (IQR: 4.2-6.9) for men. The mean (SD) age at baseline was 72 (4) years for women and 72 (5) years for men. Women had lower baseline femoral neck BMD than men, 0.66 g/cm² (95% CI: 0.65, 0.66) in women and 0.79 g/cm² (95% CI: 0.78, 0.79), $P<0.001$ (Table 3.1).

On average, women lost 0.63% (SD: 0.70%) and men lost 0.37% (0.57%) of femoral neck BMD per year. The rate of BMD loss increased with age in both genders ($P < 0.001$). For women, the mean annual rate of bone loss was: -0.52% (95% CI: -0.55 -0.49) for ages 65-69, -0.62% (95% CI: -0.65, -0.59) for ages 70-74, -0.72% (95% CI: -0.77, -0.68) for ages 75-79, and -0.95% (95% CI: -1.0, -0.88) for ages 80+. In men, the rate was: -0.31% (95% CI: -0.34, -0.28) for ages 65-69, -0.35% (95% CI: -0.39, -0.32) for ages 70-74, -0.42% (95% CI: -0.47, -0.38) for ages 75-79, and -0.55% (95% CI: -0.62, -0.49) for ages 80+ (Figure 3.2A).

3.3.2 Smoking and FNBMD change

At baseline, 7.8 % of women and 2.9% of men self-reported as current smokers. Current smoking was associated with increased bone loss in both genders (Figure 3.2B). In women, current smokers had the highest annual bone loss rate (-0.77% [95% CI: -0.84 -0.70] compared to past smokers (-0.61% [95% CI: -0.65 -0.58]) and non-smokers (-0.63% [95% CI: -0.65 -0.60]), and this difference remained significant after adjusting for covariates ($P<0.001$). In men, current smokers tended to have a higher rate of bone loss (-0.53% [95% CI: -0.66, -0.40]) compared to past smokers (-0.35% [95% CI: -0.38, -0.33]) and non-smokers (-0.39% [95% CI: -0.42, -0.36]). However, these differences did not reach statistical significance under the defined threshold ($P = 0.037$ and $P = 0.016$, respectively).

Table 3.1 Characteristics of study participants

Baseline characteristics	**Women N = 5,288**	**Men N = 3,557**	**P-value**
Age (yrs)	72 (4)	72 (5)	0.706
Weight (kg)	66.8 (11.4)	83.6 (12.8)	0.000
Height (cm)	160 (5.6)	175 (6.7)	0.000
BMI (kg/m^2)	26.2 (4.2)	27.3 (3.7)	<0.001
Femoral neck BMD (g/cm^2)	0.66 (0.11)	0.79 (0.13)	0.000
Smoking status			<0.001
Smoker	413 (7.8 %)	105 (2.7%)	
Ex-smoker	1601(30.3 %)	2044 (57.5%)	
Non-smoker	3274 (61.9%)	1408 (39.6%)	
Drinking status			<0.001
0 drink/week	2170 (41.1 %)	1109 (31.2%)	
<1 drink/week	1400 (26.5 %)	457 (12.8%)	
≥1-<7 drinks/week	1121 (21.3%)	1033 (29.0%)	
≥7-<14 drinks/week	414 (7.82%)	543 (15.3%)	
≥14-<21 drinks/week	140 (2.65%)	278 (7.82%)	
≥21 drinks/week	43 (0.81%)	137 (3.85%)	
Dietary calcium intake (mg/day)	1243 (630)[a]	1150 (586)	<0.001
Dietary protein intake (g/day)	50.9 (13.7)[b]	56.5 (17.3)[b]	<0.001
Physical activity (kcal/day)	1827 (1070)[c]	2258 (455)[c]	<0.001

Values are presented as mean (SD) unless otherwise specified. [a] Only 4,246 women with total calcium intake were included. [b] Only 2,915 women and 2,897 men with dietary protein intake were included. [c] Only 3,605 women and 3,114 men with physical activity data were included in the analysis.

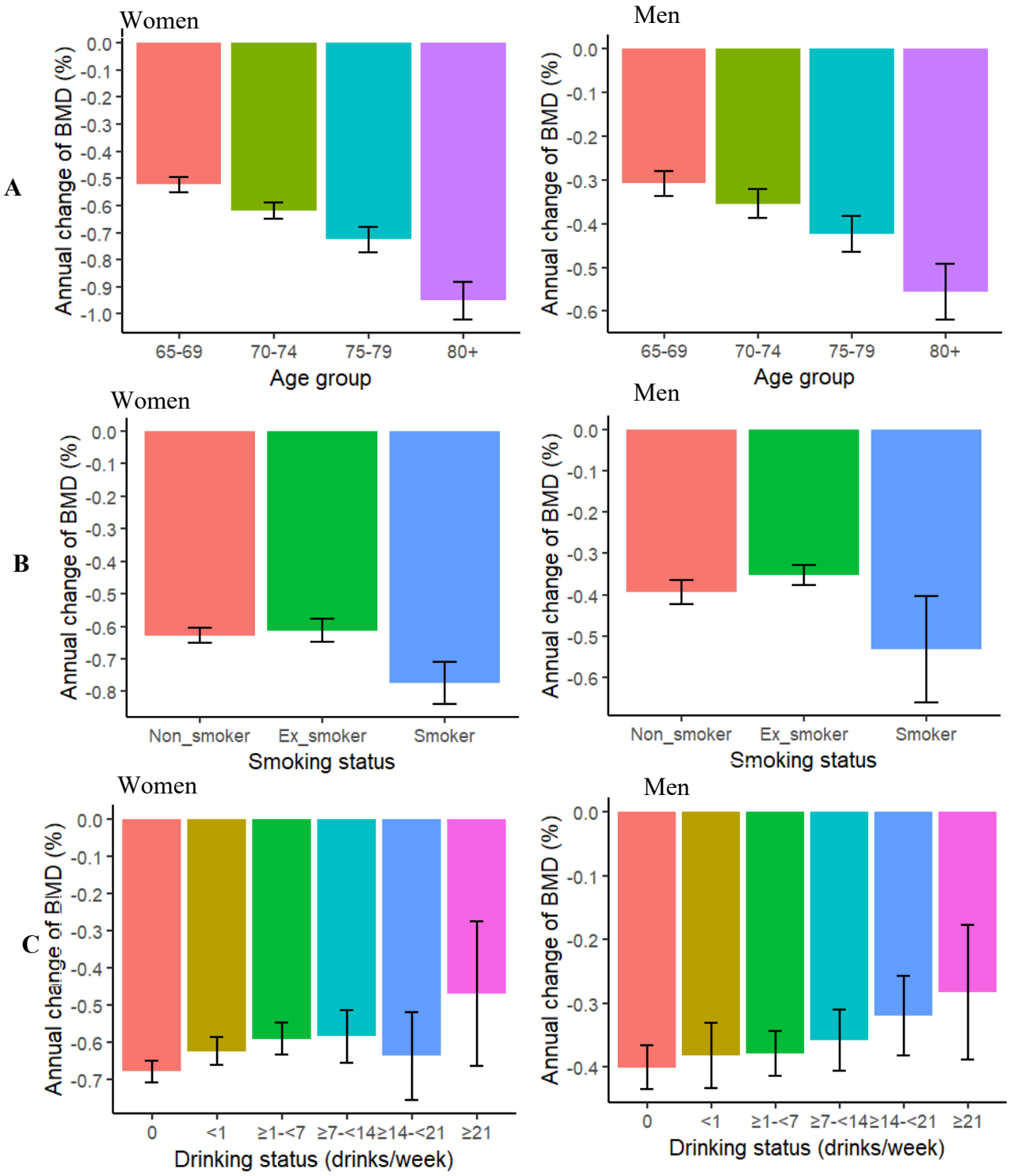

Figure 3.2 Annual percentage change in bone mineral density (BMD) at femoral neck stratified by age groups (**A**) and smoking status **(B)**, and by drinking status **(C)** in 5,288 women and 3,557 men.

3.3.3 Alcohol intakes and BMD change

Approximately 59% of women and 69% of men self-reported as current drinkers. In women, non-drinkers had a mean bone loss of -0.68% (95% CI: -0.71, -0.65). This bone loss decreased to -0.59% (95% CI: -0.63, -0.55) for those consuming 1 to <7 drinks/week, and this reduction was statistically significant (*P*-adjusted <0.001). In men, non-drinkers experienced a bone loss of -0.40% (95% CI: -0.43, -0.37), with reductions to -0.32% (95% CI: -0.38, -0.26) for those consuming 14 to <21 drinks/week. However, the difference was not statistically significant (*P*=0.14, Figure 3.2C).

3.3.4 Dietary calcium and protein intakes and BMD change

Average total calcium intake was lower in men (1150 mg/day [586]) than in women (1243 mg/day [630]). Figure 3.3A shows a significant relationship between total calcium intake and the rate of bone change in women but not in men. Among women, those consuming 698 mg/day or less had the highest rate of femoral neck BMD loss (–0.70% per year [SD = 0.69]) compared to those consuming 1199–1719 mg/day (–0.55% [SD = 0.77], P-adjusted < 0.001) and those consuming ≥1720 mg/day (–0.53% [SD = 0.70], P-adjusted < 0.001). The difference was not statistically significant between the lowest intake group and those consuming 699–1198 mg/day (–0.63% [SD = 0.66], P = 0.04). Among men, those consuming 687 mg/day or less experienced a greater rate of BMD loss (–0.40% [SD = 0.60]) than those consuming ≥1573 mg/day (–0.35% [SD = 0.57], P = 0.038), though the effect size was modest (Figure 3.3A).

Average dietary protein intake was lower in women, averaging 50.9 (SD 13.7) grams per day compared to men, who averaged 56.5 (SD 17.3) grams per day. No significant correlation was found between dietary protein intake and changes in femoral neck BMD in women or men (Figure 3.3B).

3.3.5 Physical activity and BMD change

Women had lower physical activity levels, measured in kcal/day, than men. Figure 3.3C shows the association between total kilocalories and femoral neck BMD change. Women with the second highest activity level (1539–2497 kcal/day) experienced less bone loss (-0.59% [SD: 0.7]) compared to those with the lowest activity levels (≤954 kcal/day), who had a bone loss rate of -0.64% [SD: 0.69] per year. Women with the highest activity level (≥2498 kcal/day) had a bone loss rate of -0.61% [SD: 0.71] per year. However, this association was not statistically significant (P = 0.43).

In men, there was a statistically significant association between total physical activity (kilocalories) and femoral neck BMD change (P < 0.001). Men with the highest activity level (≥2521 kcal/day) had the lowest bone loss rate of -0.29% [SD: 0.47] compared to -0.50% [SD: 0.65] in those with the lowest activity level (≤1944 kcal/day). For men with intermediate activity levels, bone loss rates were -0.38% [SD: 0.56] for 1945–2218 kcal/day and -0.31% [SD: 0.51] for 2219–2520 kcal/day (P-adjusted < 0.001).

Relative importance analysis showed age, smoking habit, alcohol use, total calcium intake, dietary protein intake, and physical activity explained 4.1% and 4.0% of BMD rate variance in women and men, respectively (Figure 3.4). The most important contributor was age in women and physical activity in men, contributing to ~41.5% (i.e., 1.7% of total 4.1%), and ~57.5% (2.3% of total 4.0%) of BMD change variance explained by the model (Table 3.2).

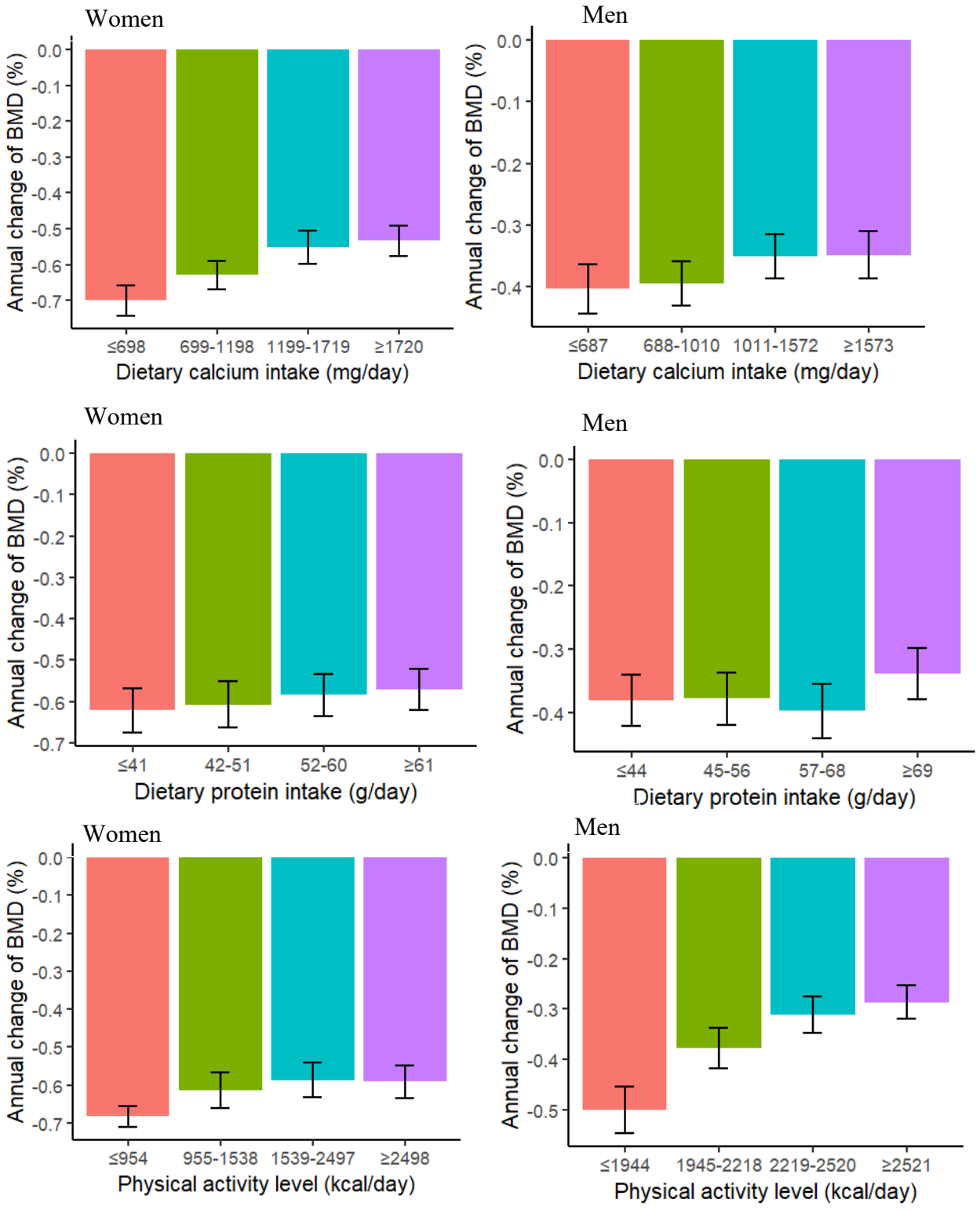

Figure 3.3 Annual percentage change in bone mineral density (BMD) at the femoral neck by quartile of total calcium intake (A) in 4246 women and 3,557 men, and dietary protein intake (B) in 2,915 women and 2,897 men, and by physical activity level (C) in 3,605 women and 3,114 men.

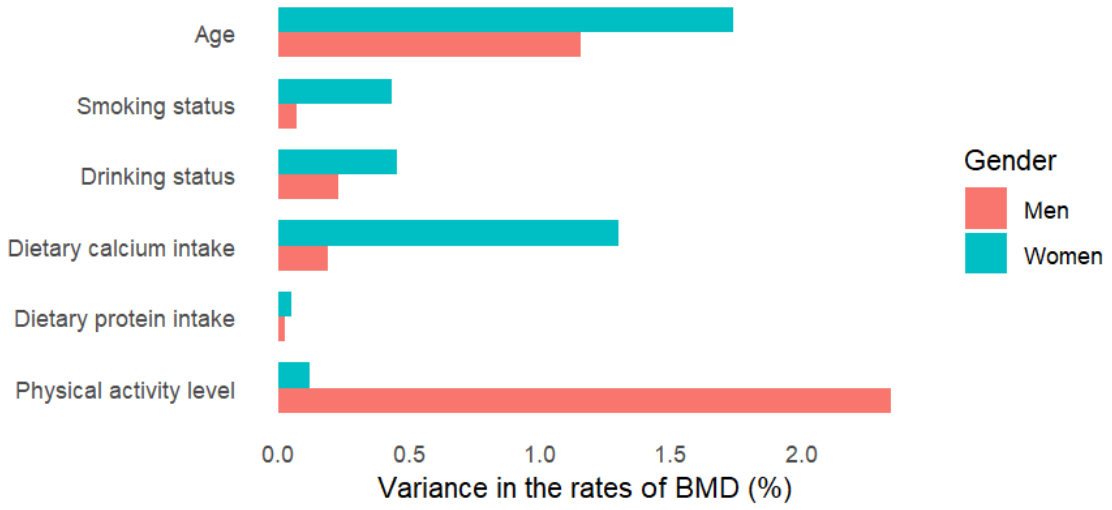

Figure 3.4 The percentage of variance in the rate of bone mineral density (BMD) in femoral neck contributed by age, smoking status, drinking status, dietary calcium intake, protein intake, and physical activity level in 1,940 women and 2,543 men. Age was in years. Non-smokers were used as a reference for smoking status, and 0 drink/week was used as a reference for drinking status. Total calcium intake, dietary protein intake and physical activity were measured in a log scale.

Table 3.2 Contributions of lifestyle factors to variance of bone change in older women and men

Predictive factor	Unit	Women (n=1,940)			Men (n=2,543)		
		Regression coefficient (standard error)	P-value	Percent of contribution to variance in bone loss	Regression coefficient (standard error)	P-value	Percent of contribution to variance in bone loss
Age	1 year	-0.024 (0.004)	<0.001	**1.7%**	-0.01 (0.002)	<0.001	**1.6%**
Smoking	current vs. never	-0.217 (0.07)	0.001	**0.4%**	-0.06 (0.06)	0.30	**0.07%**
Alcohol	Drinking* vs. never	0.073 (0.04)	0.09	**0.5%**	0.075 (0.04)	0.04	**0.23%**
Dietary calcium intake**	ln(mg/day)	0.140 (0.03)	<0.001	**1.3%**	0.044 (0.02)	0.02	**0.2%**
Dietary protein intake**	ln(g/day)	-0.037 (0.06)	0.53	**0.05%**	-0.04 (0.03)	0.2	**0.03%**
Physical activity**	ln(kcal/day)	0.032 (0.03)	0.25	**0.12%**	0.31 (0.05)	<0.001	**2.3%**

Note: *Drinking status comparing ≥1-<7 vs never in women, and ≥14-<21 vs never in men; ** in natural logarithmic scale.

Black women had significantly higher average age and BMI compared to White women, at 75 (3) years and 30 (4.2) kg/m^2, respectively. On average, they lost 0.37% (SD: 0.76%) of femoral neck BMD per year, with the rate increasing with age: -0.28% (95% CI: -0.39, -0.16) for ages 70-74, -0.38% (95% CI: -0.51, -0.25) for ages 75-79 (*P*=0.28), and -0.76% (95% CI: -1.13, -0.40) for ages 80+ (*P*-adjusted=0.001) (Figure S3.3A). At baseline, about 7.2% of Black women were current smokers. Smokers had the highest annual bone loss rate (-0.45% [0.78]) compared to past smokers (-0.37% [0.80%]) and non-smokers (-0.36% [0.76]), though this difference was not statistically significant (*P*=0.87, Figure S3.3B). Approximately 30.5% reported as current drinkers, with no significant association with bone loss (*P*=0.55). The average total calcium intake was lower in Black women compared to White women (Table S3.2), (920 [556] vs 1243 [630] mg/day, P<0.001), while the average dietary protein intake was similar (50.9 [13.7] vs 51.4 [16.7] mg/day). No significant correlations were found between dietary intake and changes in femoral neck BMD. Relative importance analysis indicated that age, smoking habit, alcohol use, total calcium intake, dietary protein intake, and physical activity together explained 16.7% of the variance in BMD rate among Black women. Alcohol use and age were the largest contributors, accounting for approximately 44.9% (7.5% of the total 16.7%) and 14.3% (2.4% of the total 16.7%), respectively. Dietary protein intake contributed 17.9% (3.0% of the total 16.7%; Figure S3.4).

3.4 Discussion

Lifestyle factors, dietary calcium intake, and physical activity are associated with variation in BMD, but their impact on longitudinal rates of bone loss remains unclear. This study offers valuable insights: (1) cigarette smoking was linked to an accelerated femoral neck BMD loss in women but not in men, while moderate alcohol intake was associated with a slower rate of bone loss in both sexes; (2) higher physical activity was associated with reduced femoral BMD loss in men but not in women; and (3) higher total calcium intake was associated with reduced femoral bone loss in women but not in men, while total dietary protein intake had no apparent effect. Despite statistical significance, these factors

collectively explained only a small fraction (less than 4.2%) of the variance in bone loss. This low R^2 value is expected given the complex, multifactorial nature of bone loss, which is influenced by genetic predisposition, hormonal status, and other environmental and physiological factors not captured in this analysis. Moreover, bone density measurements carry intrinsic variability, particularly at lower BMD values, which may further reduce the proportion of variance explained. Nonetheless, even small individual-level effects may correspond to meaningful impact at the population level, consistent with the concept of Rose's prevention paradox [225].

The mean rate of femoral neck bone loss in this study was -0.63% per year in older women and -0.37% per year in older men. Our findings are consistent with previous studies demonstrating a greater rate of bone loss at the femoral neck with advancing age [226, 227]. Comparable rates were observed in the Dubbo Study, with the rate of femoral neck bone loss ranging from -0.64% to -1.28% per year in women and from -0.52% to -1.28% per year in men [226], and in the Rotterdam Study, where the annual femoral neck bone loss was approximately -0.6% [228]. While previous studies suggested a relatively constant rate of age-related bone loss [229], our findings indicate that the rate of bone loss steadily increases with advancing age. Although the rate of bone loss increases with age, we did not formally test for acceleration (i.e., change in the rate of loss) using age-squared terms. Thus, our findings reflect a stepwise increase in loss across age groups rather than statistical acceleration.

We found that current smoking was associated with greater BMD loss at the femoral neck in women compared to those who never smoked and past smokers, but it had no clear effect in men. This finding contrasts with some studies showing increased bone loss with smoking in both older women and men [228, 230], possibly because those studies focused on younger populations where smoking may have a more significant impact than in an older population. The Dubbo study also found no clear relationship between current smoking and bone loss in older men [226]. Another possibility for this finding is the small number of smokers in our sample (n=105) compared to non-smokers (n=1408) and ex-smokers (n=2044), which may have limited the power to detect differences in men.

Previous studies provided evidence suggesting smoking increases bone resorption, as indicated by elevated bone markers in heavy smokers [231]. Our observations align with studies finding no significant difference between former smokers and non-smokers [206, 232]. Overall, our findings suggest smoking cessation may be associated with a lower rate of bone loss, particularly in older individuals.

Our analysis showed a trend of decreasing femoral neck bone loss with increasing alcohol consumption. Specifically, women who consumed 1-7 drinks per week had a significantly lower rate of bone loss compared to non-drinkers. A similar trend was observed in men who consumed 7-14 drinks per week, though the significance was less pronounced than in women. The lowest rates of bone loss were observed in the highest alcohol consumption categories, although these differences were not statistically significant. This is consistent with prior studies reporting reduced bone loss among postmenopausal women consuming alcohol at least once a week [233] and men drinking more than one drink per week [228, 234], adding to evidence that moderate alcohol consumption may be associated with better bone health [235].

However, it is important to note the potential for confounding in this association. While we adjusted for multiple covariates, the possibility of residual confounding remains, since individuals who abstain from alcohol may differ from drinkers in various sociodemographic and lifestyle factors, such as diet, physical activity, and overall health status, which could influence bone loss independent of alcohol intake.

Our findings suggest that moderate alcohol consumption may be linked to a lower rate of bone loss, possibly due to its influence on bone remodeling rather than a direct causal effect. Studies have found that alcohol drinkers had lower levels of bone resorption markers, such as urinary cross-linked N-terminal telopeptides of type I collagen to creatinine (NTX:Cr), serum osteocalcin, and serum parathyroid hormone (PTH), which are markers of bone resorption [236]. Additionally, moderate drinkers exhibited suppressed bone resorption, as indicated by lower levels of serum carboxy-terminal telopeptide of type I collagen (CTX) [237]. While these findings contribute to the understanding of the

impact of alcohol on bone health, the relationship remains complex and requires further study to account for potential confounders and establish causality.

The role of dietary protein intakes in bone health remains a contentious issue, with evidence suggesting that different protein sources may have varying effects on bone metabolism. We observed a trend similar to that found by Langsetmo et al, who studied a Canadian population and reported that higher protein intake was associated with greater femoral neck BMD in both men and women [238]. Additionally, previous studies in the MrOS cohort have examined the relationship between protein intake and bone health, distinguishing between dairy, non-dairy animal, and plant protein sources [219, 239]. However, we found no significant association between total dietary protein intake and bone loss, consistent with the findings of Rapuri et al., which also reported no correlation between total dietary protein intake and bone loss [240].

In contrast, other studies have reported an association between low protein intake and increased bone loss. For example, Han et al. found that individuals consuming an average protein intake of around 500 g/day experienced lower bone loss rates [212], and a positive correlation between dietary protein intake and bone change rate [241]. However, it is important to recognize that not all protein sources have the same effect on bone health. The studies by Langetmo et al. have highlighted that dairy protein intake, in particular, is associated with greater bone strength parameters at the distal radius and tibia in older men [219], while non-dairy animal and plant proteins may have different effects [239].

The discrepancies between our findings and those of other studies may be attributed not only to differences in the levels of dietary protein intake among study populations but also to differences in the composition of protein sources (e.g., dairy, non-dairy animal, and plant protein). Previous research suggests that plant protein can provide all essential amino acids when consumed in sufficient variety [242, 243]; however, there are notable differences between North American and other global diets in terms of plant protein consumption, with some dietary patterns potentially providing incomplete protein profiles. These differences in protein quality, along with variations in measurement methods, may have affected our ability to detect an association between protein intake and

bone loss in this study. Future studies should further examine the differential effects of protein sources on bone health to provide a more nuanced understanding of protein's role in maintaining bone health.

For total calcium intakes, we found an association with bone loss in women, but not in men, consistent with other studies in women [244-246] while failing to establish a clear association in men [247]. Although the role of calcium has been debated, collective evidence from observational studies and randomized controlled trials suggests that calcium has a small but significant effect on bone health [245, 246], consistent with our findings. While some studies reported no association in women [248], our results align with the broader literature indicating that calcium intake contributes to bone health, albeit with a modest effect size. Our study could not confirm a protective effect of calcium intake on bone loss in older men, nor an association between protein intake and bone loss in older women and men. The use of quartiles to categorize continuous variables such as dietary protein intake may obscure nonlinear relationships or dilute extreme exposure effects. This approach was chosen due to skewed distributions and to enable consistent comparisons, but future studies may consider spline or dose-response modeling to explore thresholds and nonlinear effects more precisely.

We observed lower rates of bone loss in physically active women and men compared to those with low activity levels. The protective effects of physical activity on bone health are well-established, with substantial evidence supporting its role in reducing bone loss. This aligns with previous studies showing minimal bone loss in active older individuals, while less active and sedentary women experienced higher bone loss [227, 249]. Another study reported that increased training or exercise was associated with a 1% increase in bone density [250]. Notably, our findings suggest that physical activity is among the most significant modifiable factors associated with differences in bone loss, with a stronger effect compared to other lifestyle factors such as dietary calcium or protein intake. These findings underscore the importance of regular physical activity for maintaining bone health.

It is important to note that a combination of age, smoking status, alcohol consumption, total calcium and dietary protein intake, and physical activity collectively contributed to approximately 4.1% of the variance in femoral neck bone loss, with advancing age accounting for 1.7% in women and physical activity 2.3% in men. Given that bone loss is inherently measured with some degree of error, it is expected that the explained variance remains modest. Additionally, genetics factors [133, 251], other lifestyle and environmental factors, and hormonal factors [227] also contribute to bone loss, further limiting the explanatory power of lifestyle factors alone. Although the small effect sizes imply limited individual-level benefits, the Rose prevention paradox suggests that modest sifts in BMD across the population can yield substantial reductions in hip fractures incidence [225]. Thus, while lifestyle factors explain only a small fraction of bone loss variance, their cumulative impact on fracture prevention at the population level remains significant.

This study has many strengths that enhance its validity and reliability. Our findings provide valuable insights into how multiple lifestyle factors collectively influence bone loss, with a particular emphasis on the dominance of physical activity over dietary factors. The study utilized two large, well-established cohorts, enhancing estimate precision. The longitudinal design with extended follow-up allowed precise assessment of individual rate of bone change. The utilization of linear mixed-effects regression analysis robustly addressed the issue of clustered repeated measurements taken from the same individuals over time. Unlike conventional linear regression, which assumes independence of observations, linear mixed-effects models incorporate random effects to account for within-subject variability and effectively model time-varying covariates.

However, our findings should be interpreted with potential limitations in mind. We recognize that lifestyle behaviours may change over time and that relying on baseline measures may not fully capture their cumulative effects on BMD. However, this decision was made to ensure consistency and comparability across SOF and MrOS, which had different follow-up visit schedules and availability of exposure data. Although we included comorbidities and medications, other potential contributors to bone loss, such as

environmental factors like household conditions and air quality, as well as vitamin D intake, were not evaluated. Categorizing smoking and alcohol consumption as categorical rather than continuous variables may have underestimated their influence on bone loss. The effects of heavy drinking, alcoholism, or the quantity of cigarettes smoked on bone loss were not investigated. The assessment of total calcium and dietary protein intakes, as well as physical activity levels, relied on self-reported data, which may have introduced potential recall and reporting biases. While dietary calcium intake may serve as a proxy for dairy intake—often accompanied by protein, vitamin D, and other nutrients—the observed association may not be solely attributable to calcium. Additionally, while we adjusted the association between total calcium and dietary protein intake for BMI, we did not adjust for total energy intake. This omission may have influenced the observed associations when accounting for differences in body size. Furthermore, some alcohol consumption categories, particularly at higher intake levels, had smaller sample sizes, which may have reduced statistical power and affected our ability to detect significant differences between groups. Although efforts were made to align measurement timepoints between SOF (Visit 6) and MrOS (Visit 1), some differences in cohort design and data collection timing remain, which could contribute to residual inconsistencies in the comparability of lifestyle exposures. Finally, the study populations were generally community-dwelling, healthier individuals, potentially limiting generalizability to other ethnicities and health profiles.

In conclusion, our study indicates that maintaining moderate physical activity and alcohol consumption, while abstaining from smoking, may be associated with a slower rate of age-related bone loss and reduce osteoporosis risk among older women and men. Future research could examine the dose-response relationships between alcohol consumption and bone health and identify optimal physical activity levels for various age groups and genders.

CHAPTER 4.

AGE-RELATED BONE LOSS AND SUBSEQUENT MORTALITY IN ELDERLY MEN AND WOMEN

4. Age-Related Bone Loss and Subsequent Mortality in Elderly Men and Women

4.1 Introduction

Bone loss occurs when bone resorption surpasses bone formation, and the resulting condition, osteoporosis, has become a significant public health concern among elderly men and women worldwide. Beyond low bone mineral density (BMD), bone loss contributes to an elevated risk of fragility fractures, which themselves increase the likelihood of subsequent fractures and are linked with higher mortality rates [252-254]. Therefore, bone loss may serve as an indicator for overall declining health and elevated mortality risk in the general population [203, 255]. In a study by Kado et al. based on 6,046 participants in the United States, each 1 SD increase in bone loss at the hip was found to be associated with a 30% higher risk of overall mortality. A similar pattern was observed in Australia, where Nguyen et al. reported that low femoral neck BMD (FNBMD), alongside weight loss and weight fluctuation, served as independent predictors of mortality risk, increasing mortality risk by 22% in women, and 36% in men [255].

Previous studies, including those utilizing the well-characterized MrOS and SOF cohorts with extended follow-up periods, have explored the relationship between bone loss and mortality in both men and women. However, these studies had limitations due to variations in measurement sites, reliance on standard deviation decreases to compare bone loss—which may not be fully generalizable across populations—and differing methods for calculating bone loss. To address these limitations, this study aims to examine the relationship between bone loss rates and mortality risk by employing consistent BMD measurements at the femoral neck and conducting more frequent assessments over time. Utilizing a robust mixed-effects model, this approach accounts for the variability in individual bone loss trajectories across multiple BMD measurements, offering more precise, reproducible, and clinically relevant insights. Unlike earlier studies, our approach provides a unique perspective, adding a novel contribution to the understanding of osteoporosis and its broader health impacts.

4.2 Material and Methods

This study utilized data from two large prospective cohort studies: the Study of Osteoporotic Fractures (SOF) for women and the Osteoporotic Fractures in Men (MrOS) for men. Further details on the SOF and MrOS study designs and protocols have been described in Chapter 2.

The analytical sample was restricted to participants with at least three BMD measurements to ensure reliable estimation of bone loss (n = 5,343 women and 3,577 men) (Figure 4.1). To minimize immortal time bias, only participants with sufficient follow-up to yield at least three BMD values were included. Bone loss was modelled using data from these follow-up visits. However, because survival analyses were initiated from baseline, the potential for immortal time bias cannot be excluded. Future studies may consider joint modeling approaches to more rigorously address this limitation.

The analysis focused on femoral neck BMD rather than lumbar spine BMD, as the femoral neck is a weight-bearing site with high clinical relevance for fracture risk, and is less affected by degenerative changes that may compromise spine measurements.

A descriptive analysis was conducted to examine differences in bone loss rate and mortality. Each participant's rate of BMD change was estimated using a linear mixed-effects regression model, with BMD values plotted against time. The annual rate of bone change (%/year) was derived by dividing the estimated slope by baseline BMD and multiplying by 100.

Rates of bone change were categorized into four groups based on individual annual trajectories: "unchanged" (-1% to 1%), "increased" (>1%), "decreased" (-1% to -2%), and "rapidly decreased" (<-2%). The term "rapid" in this context refers to a threshold-based categorization using linear slopes and does not reflect non-linear modeling of BMD change (e.g., using age^2 terms), but serves as a clinically relevant stratification. Bone loss was also analyzed as a continuous variable (per 1 SD decrease).

Cox proportional hazards models were used to evaluate the association between bone loss (both categorical and continuous) and mortality risk, adjusting for predefined covariates: age, BMI, baseline femoral neck BMD, smoking status, alcohol use, osteoporosis medication use, and comorbidities. For men, ethnicity was also included as a covariate.

Follow-up time was calculated from the date of the first BMD measurement—Visit 1 for MrOS and Visit 2 for SOF—until death or censoring at the study end date (December 31, 2016, for MrOS and April 5, 2019, for SOF), whichever occurred first. Baseline BMD was included in the Cox models for mortality as an independent predictor, but not in the estimation of BMD change. This approach aligns with the recommendations of Glymour et al., which emphasize the importance of context when deciding whether to adjust for baseline values [256].

All covariates were measured at baseline and treated as fixed. Time-varying changes in exposures during follow-up were not incorporated. Statistical analyses were conducted using R version 3.5.1, and a two-sided P-value < 0.05 was considered statistically significant.

This study also serves as a methodological foundation for a planned extension using the concept of skeletal age, a clinically meaningful metric to communicate mortality risk. Inspired by frameworks such as "heart age" and "effective age" [Spiegelhalter, 2016], skeletal age represents the biological age of an individual's skeleton based on their fracture and mortality risk profile. A key principle is that elevated hazard ratios (HRs) for mortality—such as those observed with accelerated bone loss—can be translated into years of life lost, using the formula:

$$\text{Skeletal age difference} = \frac{\log(HZ)}{\log(1.1)}$$

This expression reflects the exponential increase in mortality with aging, where the annual hazard roughly increases by 10% per year [257]. While skeletal age estimation is not the primary outcome of this chapter, the mortality risk estimates derived here provide the foundation for its future application.

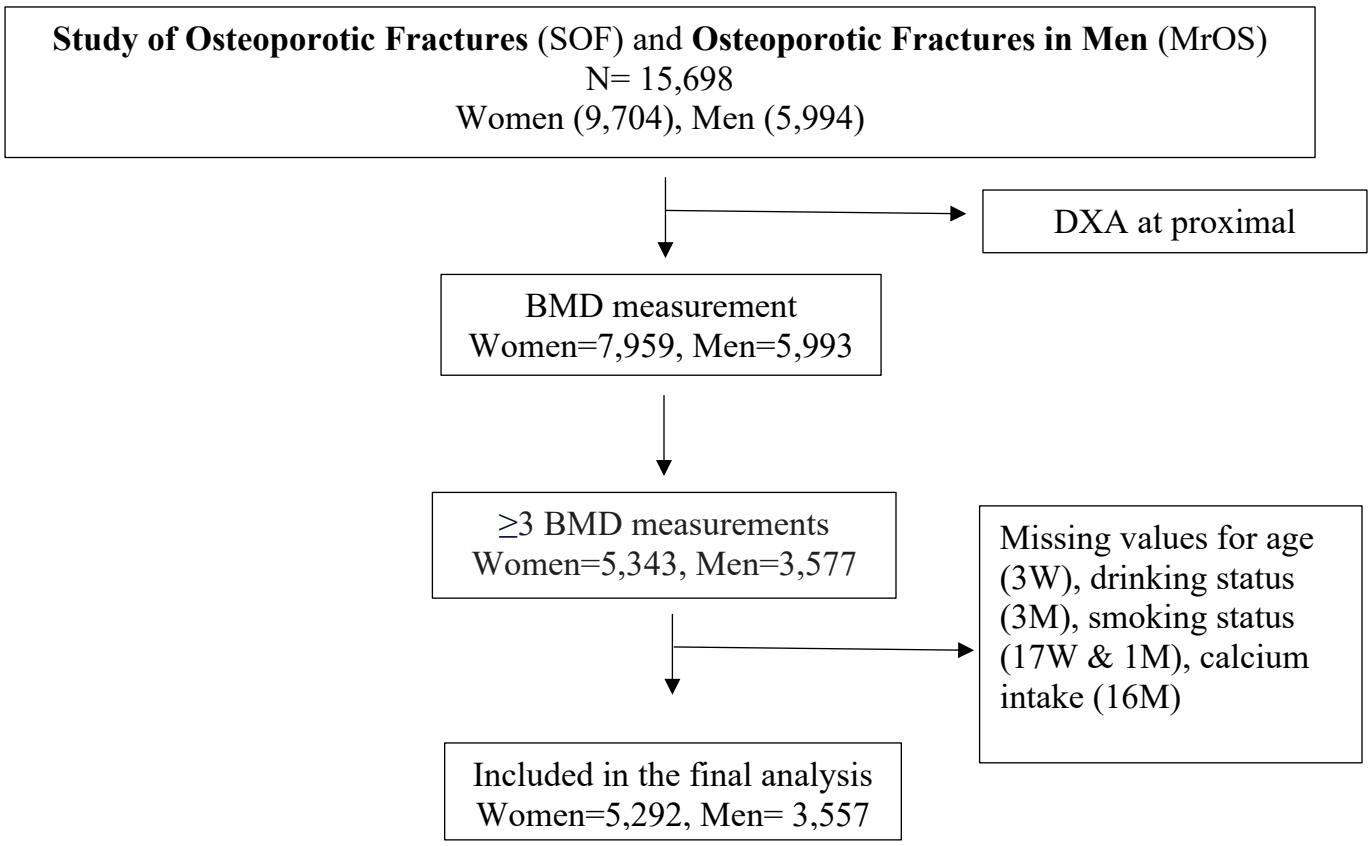

Figure 4.1 Flow chart of SOF and MrOS participants according to the analytical samples. Abbreviations: BMD, bone mineral density; M,men; W, women.

4.3 Results

4.3.1 Baseline characteristics

The present analysis included 8,849 participants (3,557 men and 5,292 women), each with at least 3 BMD measurement over a median follow-up of 16.9 year (IQR:12.6, 20.2) in men and 17 years (IQR:13.0, 19,5) for in women, 2,781 men and 2,746 women had died. Men who died were older, shorter, had lower baseline BMD at femoral neck, and were more likely to be a current smoker than those who survived ($P < 0.001$ for all) (Table 4.1). A similar pattern was observed for women. Compared to women who survived, those who died were older, had lower baseline BMD at femoral neck, where more likely to be current smoker ($P<0.001$ for all), and less likely to consume alcohol than those who survived ($P=0.036$).

Table 4.1 Characteristics of study participants

Characteristics	**Women**		**Men**	
	Survivors (N=2,546)	Deceased (N=2,746)	Survivors (N=776)	Deceased (N=2,781)
Age (yrs)	71 (3.5)	74 (4.7)*	69 (3.1)	73 (5.1)*
Height (cm)	160 (5.5)	159 (5.7)	176 (6.5)	174 (6.8)*
Weight (kg)	67 (11.1)	67 (11.7)	84 (12)	84 (13)
BMI (kg/m^2)	26 (4.1)	26 (4.3)	27 (3.5)	27 (3.7)
Smoking status: Current Past Never	147 (5.8%) 772 (30.3%) 1627 (63.9%)	266 (9.8%) 829 (30.1%) 1651 (60.1%)	18 (2.3%) 383 (49.4%) 375 (48.3%)	87 (3.2%) 1661 (59.7%) 1033 (37.1%)
Alcohol status: Drinker	1571 (61.7%)	1548 (56.3%)	557 (71.8%)	1891 (67.9%)
Femoral neck BMD (g/cm^2)	0.666 (0.108)	0.650 (0.107)*	0.800 (0.12)	0.788 (0.13)
Medication: User	1382 (54.3%)	1292 (47.1%)*	46 (6.3%)	200 (7.6%)
Comorbidities: At least one	1497 (58.8%)	1457 (53.1%)*	373 (48.1%)	1717 (61.7%)*

Values are presented as mean (SD) unless otherwise specified.

4.3.2 Incidence of mortality

The unadjusted mortality incidence rate was 4.7 deaths/ per 100 person-years (95% CI: 4.5, 4.9) in men and 2.9 deaths per 100 person-years (95% CI: 2.8, 3.0) in women. Importantly, the unadjusted mortality rate among participants with a rapid decrease in the rate of bone loss was higher, at 7.1 per 100 person-years in men and 5.4 per 100 person-years in women, compared to those with an unchanged rate of bone loss (4.5/100 person-years in men and 2.5/100 person-years in women). The analysis based on the rate of bone change demonstrated that both men and women who experienced a rapid decrease or decreased rate of bone change had a higher risk of mortality compared to those whose rate of bone loss remained unchanged (Table 4.2).

Table 4.2 Incidence of mortality following specific rate of bone changed stratified by gender

Rate of bone change	Number	Age (years)	FNBMD	Number of deaths	Follow-up time (person-years)	Rate* of mortality (95%CI)
Men						
Unchanged	3136	72 (5)	0.795 (0.12)	2399	52952	4.5% (4.3, 4.7)
Increased	14	71 (5)	0.762 (0.15)	10	239	4.2% (1.6, 6.8)
Decreased	387	74 (5)	0.758 (0.13)	353	5711	6.2% (5.5, 6.8)
Rapid decrease	20	74 (5)	0.729 (0.22)	19	269	7.1% (3.8, 10.2)
Women						
Unchanged	3793	72 (4)	0.671 (0.11)	1780	70098	2.5% (2.4, 2.6)
Increased	89	73 (4)	0.663 (0.16)	42	1665	2.5% (1.7, 3.3)
Decreased	1318	73.1 (4.6)	0.623 (0.10)	552	13976.9	3.9% (3.6, 4.2)
Rapid decrease	92	75 (5)	0.576 (0.13)	75	1401	5.4% (4.1, 6.6)

Note: * rates were calculated as number of deaths/100 person-years

4.3.3 Unadjusted analysis

In unadjusted models, both men and women with a rapid decrease in FNBMD had a significantly increased risk of mortality compared to those with unchanged FNBMD (Table 4.3). Women with a rapid decrease experienced a 216% increased risk of mortality (HR = 3.16; 95% CI, 2.5 to 4.0), while men had a 130% increased risk (HR = 2.3; 95% CI, 1.46 to 3.6). Those with decreased bone loss also faced high risk of mortality. Women with decreased bone loss had an 87% increased risk (HR = 1.87; 95% CI, 1.7 to 2.0), whereas men had a 70% increased risk (HR = 1.7; 95% CI, 1.56 to 2.0).

In contrast, individuals with increased bone density, regardless of sex, exhibited a similar mortality risk to those with unchanged FNBMD. For women, the hazard ratio was close to unity (HR = 0.98; 95% CI, 0.7 to 1.3), indicating no significant difference. Similarly, men with increased bone density had a hazard ratio of 0.90 (95% CI, 0.48 to 1.7), suggesting comparable mortality risks to those with stable bone density levels.

4.3.4 Multivariable analysis

Age and baseline BMD levels may confound the observed association between bone loss and mortality, as individuals experiencing rapid decreases in bone density were generally older and had lower baseline BMD. Additionally, lifestyle factors such as smoking and the use of bone-active medications, including estrogen, bisphosphonates, thyroid hormone, fluoride, calcitonin, and alendronate, further complicate these associations. Women with rapid decreases in bone density had higher smoking rates, while the use of medications that help maintain BMD could bias the observed relationship between bone loss and mortality. To address these confounders, a multivariable-adjusted Cox proportional hazards model was used, adjusting for age, baseline FNBMD, BMI, smoking and drinking status, medication use, and comorbidities. For men, the model also adjusted for ethnicity. This comprehensive model showed that a rapid decrease in FNBMD was associated with a significant increase in mortality risk—129% in women and 93% in men—compared to those with unchanged bone density (Table 4.3). Moderate bone loss was also linked to increased

mortality risk, by 55% in women and 43% in men, with all associations remaining significant after adjustment ($P < 0.001$). The inclusion of ethnicity in the model for men accounted for differences such as younger age, lower BMI, and lower drinking rates among Asian men, compared to older age and higher BMI among African American men (Supplementary Table 1)

4.3.5 Bone loss as continuous variable

When bone loss was analyzed as a continuous variable, each standard deviation (SD) decrease in FNBMD was associated with an increased risk of mortality in both women and men. In women, each SD decrease in FNBMD was linked to a 40% higher risk of mortality (HR per SD decrease = 1.4; 95% CI, 1.4 to 1.5), which was slightly attenuated in the multivariable model (HR per SD decrease = 1.29; 95% CI, 1.24 to 1.34). In men, each SD decrease in FNBMD corresponded to a 20% higher risk of mortality (HR per SD decrease = 1.2; 95% CI, 1.2 to 1.3), with a similar attenuation observed after multivariable adjustment (HR per SD decrease = 1.15; 95% CI, 1.10 to 1.20).

In the context of the skeletal age framework introduced in the Methods section, these hazard ratios can also be interpreted in terms of biological age acceleration. For example, in women with rapid BMD loss (HR = 2.29), the corresponding skeletal age difference is approximately 8.7 years [log(2.29)/log(1.1)], indicating that their bones are biologically equivalent to those of a person nearly 9 years older. Similarly, men with rapid BMD loss (HR = 1.93) would have a skeletal age approximately 6.9 years older than their chronological age.

T**able 4.3** Association between rate of bone loss as categorical variable and selected variables among men and women

				Unadjusted		Multivariable adjusted [a]	
	BMD	Rate of bone loss	Number	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Men	Unchanged	-1 to +1	3136	Reference	-	Reference	-
	Increased	≥ 1%	14	0.90 (0.48-1.70)	0.728	0.94(0.48-1.80)	0.841
	Decreased	-1% to -2%	387	1.70 (1.56-2.0)	<0.001	1.43(1.27-1.61)	<0.001
	Rapid decrease	≤ -2%	20	2.30 (1.46-3.6)	<0.001	1.93(1.18-3.17)	0.008
Women	Unchanged	-1 to +1	3793	Reference	-	Reference	-
	Increased	≥ 1%	89	0.98 (0.72-1.3)	0.89	0.89 (0.65-1.21)	0.454
	Decreased	-1% to -2%	1318	1.87 (1.72-2.0)	<0.001	1.55(1.43-1.69)	<0.001
	Rapid decrease	≤ -2%	92	3.16 (2.51-4.00)	<0.001	2.29(1.81-2.90)	<0.001

The association between different rate of bone change and mortality are presented as mean difference (95% confidence interval [CI] derived from a linear.

[a] Adjusted for age, baseline FNBMD, BMI, drinking status, smoking status, medication use, and comorbidities for women, with additional adjustment for ethnic groups in men.

Table 4.4 Association between rate of bone loss as continuous variable and selected variables among men and women [a]

Variable (unit)	Men (3557, 2781) [d]		Women (5292, 2746) [d]	
	HR (95%CI)	*P*-value	HR (95%CI)	*P*-value
Bone loss (per SD increase) [b]	1.15 (1.10-1.20)	<0.001	1.29 (1.24-1.34)	<0.001
Baseline FNBMD (per SD decrease) [c]	0.99 (0.95-1.00)	0.719	0.99 (0.95-1.04)	0.769
Age (per 5 year increase)	1.78 (1.71-1.80)	<0.001	1.81 (1.73-1.89)	<0.001
BMI (per SD decrease)	1.10 (1.06-1.10)	<0.001	1.04 (1.00-1.09)	0.043
Current smoker (vs. past/never)	1.61 (1.29-2.00)	<0.001	1.76 (1.55-2.00)	<0.001
Drinker (vs. non-drinker)	0.95 (0.87-1.00)	0.202	0.89 (0.82-0.96)	0.001
Medication user (vs. non-user)	1.01 (0.87-1.20)	0.933	0.88 (0.81-0.95)	<0.001
Comorbidities at least one (vs. non-comorbidities)	1.32 (1.21-1.40)	<0.001	0.76 (0.70-0.82)	<0.001
Caucasian (vs. African American/Asian/Others)	1.11 (0.96-1.30)	0.151	-	-

[a] Adjusted for age, baseline FNBMD, BMI, drinking status, smoking status, medication use, and comorbidities for women, with additional adjustment for ethnic groups in men.
[b] Number of participants with measurement of bone loss, number who deceased during follow-up
[c] Bone loss at the femoral neck for men (SD= 0.49 gm/cm^2/per year); in women (SD= 0.68 gm/cm^2/per year).
[d] Bone density at the femoral neck for men (SD= 0.128 gm/cm^2); in women (SD= 0.108 gm/cm^2).
[e] BMI for men (SD= 3.67 gm/cm^2); in women (SD= 4.23 gm/cm^2).

4.4 Discussion

In our study, we found that a rapid decrease in the rate of bone change was associated with an increased risk of mortality in both men and women, regardless of age, baseline BMD, BMI, smoking, drinking status, medication use and comorbidities. While men and women with an increased rate of bone change at the femoral neck had a similar risk of mortality as those with an unchanged rate of bone change, rather than a reduced risk of mortality.

Although bone loss was observed in both older men and women, women had a greater rate of loss than men. This finding is consistent with previous studies [202, 255, 258]. Furthermore, we found the decreased rate of bone (-1% to -2%) was associated with 1.6-fold increased risk of mortality in women and 2.4-fold increased risk of mortality in men. The strength of the association between the rate of bone change and mortality in men and women in this study was comparable with earlier studies. A longitudinal study from Nguyen et al. among 1,703 older Australian women and men in the Dubbo Study found that bone loss of 1%/yr or above increased the 1.3-fold mortality risk in both men and women [255]. Another study by Cawthon et al. in the same MrOS cohort, but using a different dichotomous assessment of bone loss, found an increased risk of mortality by 44% in men with accelerated BMD loss [202]. Similarly, a different study based on Canadian population found rapid bone loss was associated with more than a 2-fold increased mortality risk compared with no loss [259]. Our findings indicate that the rate of bone loss increases mortality risk to varying extents.

In our analysis, medication use and comorbidities were treated as distinct variables, with each being assessed independently for its contribution to bone loss. Medication use refers to whether participants were taking medications, while comorbidities reflect the presence of at least one co-existing health condition. Although medications may often be prescribed to manage comorbidities, the model evaluates their effects separately. Interestingly, in women, both medication use and comorbidities were associated with a lower risk of bone loss, while in men, comorbidities increased the risk of bone loss, with no

significant effect of medication use observed. This indicates that the two variables act independently in contributing to bone loss risk in our cohort.

This study is unique in combining multiple BMD measurements over a long-term follow-up, using a mixed-effects model to provide a more accurate assessment of bone loss trajectories. This approach allows us to capture individual variability in bone loss rates and their association with mortality risk more precisely than previous studies. The requirement of at least three BMD measurements improved the precision of the bone change estimates, enabling a more nuanced understanding of how bone loss over time contributes to mortality risk. Additionally, the use of well-characterized cohorts with comprehensive mortality data further enhanced the study's ability to detect subtle differences.

Despite these strengths, several limitations should be considered. This study focused on women of Caucasian background, while the men's cohort consisted primarily of Caucasian individuals with limited representation from minority groups. This may restrict the generalizability of the findings to other ethnic populations. Additionally, the reliance on self-reported data for variables such as smoking and alcohol consumption may introduce recall bias, potentially affecting data reliability.

Moreover, while the Cox proportional hazards model is effective for estimating mortality risk based on bone loss observed during life, it assumes that covariates remain constant throughout follow-up. In our study, time-varying changes in potential confounders—such as BMI, smoking status, drinking behaviour, medication usage, and comorbidities—were not incorporated, which may have introduced residual confounding. Future studies should consider incorporating time-updated covariates to improve the accuracy of mortality risk estimation.

While we report crude incidence rates per 100 person-years for interpretability, our multivariable models adjust for age and other factors, partially accounting for the exponential increase in mortality risk with age. Nonetheless, reporting mortality as a constant rate may understate risk among the oldest old and overstate it among younger individuals.

Importantly, this mortality analysis lays the groundwork for a planned extension using the concept of skeletal age, in which an individual's BMD-related mortality risk is translated into a biologically meaningful age difference. By applying the estimated hazard ratios to an exponential aging model [257, 260], we can convey risk in terms of years of life lost—a format that may be more accessible and motivating for patients and clinicians. For example, a woman with rapid BMD loss (HR = 2.29) would have a skeletal age approximately 8.7 years older than her chronological age. In future analyses, translating mortality risk into skeletal age may enhance the communication of age-dependent hazard and support personalized clinical decision-making.

In conclusion, rapid decrease in FNBMD is a risk factor for mortality in both men and women, whereas those with unchanged or slightly increased bone density over the follow-up period showed lower mortality rates. This suggests that the process of aging does not necessarily lead to bone loss. Future studies aim to discover the biological mechanisms that contribute to both rapid bone loss and increased mortality risk from causes other than cancer.

CHAPTER 5.

ASSOCIATION BETWEEN POLYMORPHISMS IN THE COLLAGEN TYPE 1 ALPHA 1 (COLIA1) GENE AND BONE PHENOTYPES

5. Association between polymorphisms in the collagen type 1 alpha 1 (*COLIA1*) gene and bone phenotypes

5.1 Introduction

Osteoporosis is one of the most common musculoskeletal diseases, characterized by low bone mass and deterioration of bone architecture, which leads to weakened bone strength and an increased risk of fragility fractures [255, 261]. With a lifetime fracture risk of up to 65% in women and 42% in men at the age of 60, osteoporosis poses a considerable health burden [262, 263]. For instance, in the United States alone, more than 2 million fragility fractures occur each year, with associated management costs exceeding $16 billion as of 2015 [2]. Addressing this significant healthcare burden requires more precise identification of individuals at high risk, allowing for better-targeted prevention strategies.

Genetic factors are consistently recognized as important contributors to fragility fractures [122]. Previous studies indicate that genetics may account for up to 50% of overall fracture risk in twin studies [122], and up to 64% in studies involving parental fracture history [264]. Additionally, genetic factors play a substantial role in bone mineral density (BMD) and influence BMD loss over time in elderly populations [133]. Understanding the genetic determinants of BMD and bone loss is thus essential for advancing our approach to osteoporosis prevention and treatment.

Among the various genetic contributors to osteoporosis, the collagen type I α1 gene (*COLIA1*) has been particularly well-studied. COLIA1, part of the collagen superfamily, encodes a protein critical to bone structure [135]. Research has demonstrated that a *G*-to-*T* polymorphism in the regulatory region of the *COLIA1* gene, specifically at the Sp1 binding site, is associated with BMD [136], and an increased risk of osteoporotic fractures [137, 263]. Several studies have reported links between *COLIA1* polymorphisms and both reduced bone density [125] and a higher susceptibility to osteoporotic fractures [126, 265], particularly

among postmenopausal women. These findings suggest that the *COLIA1* genotype may affect peak bone density and contribute to increased bone loss with age.

However, despite extensive research, the effect of *COLIA1* polymorphisms on BMD and bone loss remains uncertain, with conflicting results across different populations. This uncertainty underscores the need for further investigation, particularly in underrepresented cohorts. Studying bone loss is inherently complex and requires multiple BMD measurements over time to accurately observe changes. Consequently, data on the association between the COLIA1 polymorphism and the rate of bone loss is limited, especially in the context of specific populations such as Australians.

In this chapter, we aim to address these gaps by examining the effects of COLIA1 polymorphisms on BMD, bone loss, and fracture risk within a cohort of elderly Australian women aged over 60 years. This study investigates the association between COLIA1 polymorphisms and key bone phenotypes-including BMD, bone loss, and fracture risk-while controlling for factors such as age, femoral neck BMD, and other influences on bone loss, to clarify the role of COLIA1 in osteoporosis susceptibility. By utilizing multiple BMD measurements and advanced statistical modeling, this study offers a more detailed understanding of the genetic influence on bone health. This novel focus on COLIA1's role in bone loss dynamics contributes to more refined fracture risk predictions and better-targeted prevention strategies for osteoporosis.

5.2 Study design and methods

The data analyzed in this chapter were from the Dubbo Osteoporosis Epidemiology study, with the study's design and methods described in Chapter 2. In this study, we opted for femoral neck BMD measurements over lumbar spine BMD, as it is less susceptible to distortions from spinal deformities or calcifications. We included post-menopausal women who had at least three BMD measurements and available *COLIA1* data (Figure 5.1).

A descriptive analysis was conducted to examine the differences in baseline characteristics between the COLIA1 genotypes. Analysis of variance (ANOVA) was used for continuous variables, while the chi-square test was applied for categorical variables. The annual rate of BMD change for each individual was estimated using a mixed-effects linear regression.

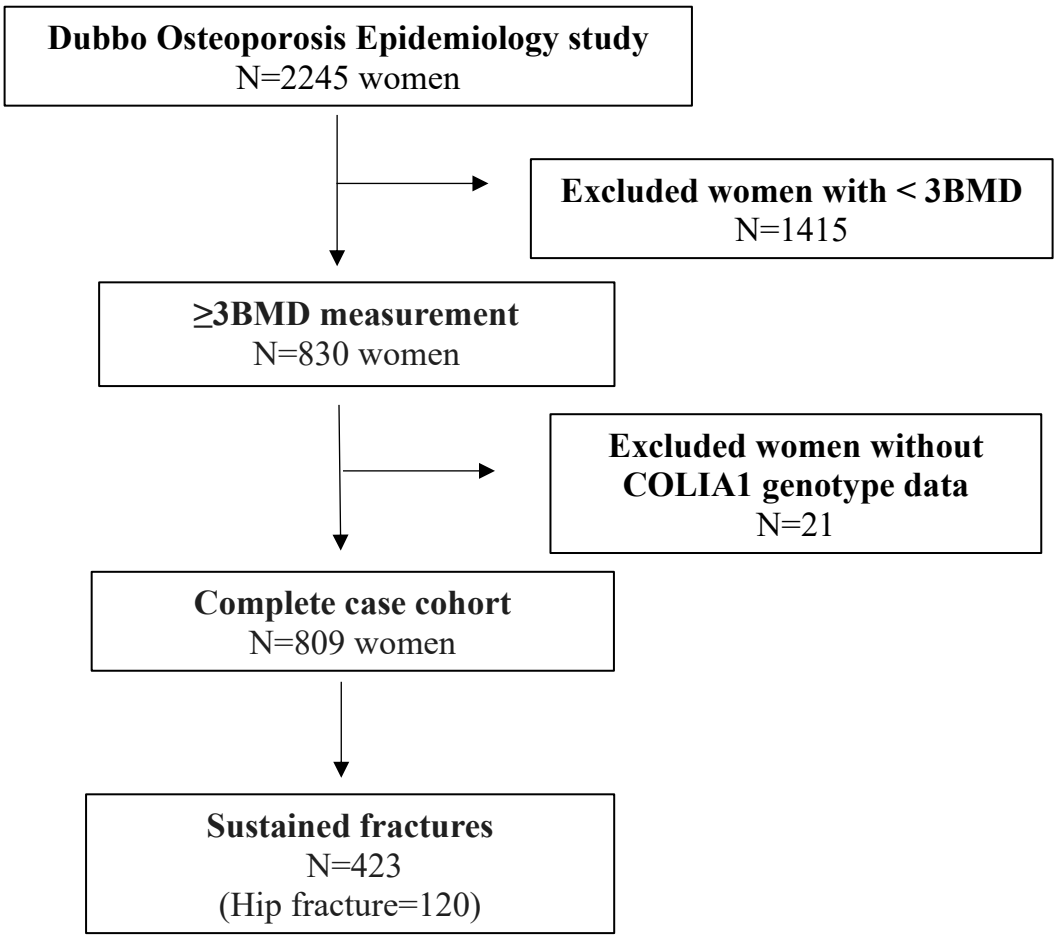

Figure 5.1 Flow chart of participants included in the Dubbo Osteoporosis Epidemiology Study Population with COLIA1 genotype from 1989 to 2021.

A mixed-effects linear regression with an unstructured covariance matrix quantified the contribution of COLIA1 polymorphisms (TT and GT versus GG) to bone loss, adjusting for predefined confounders such as age and BMI [217]. This approach was considered robust as it accounted for the correlation of repeated BMD measurements over time as well as confounding effects.

A Cox proportional hazards regression model was used to quantify the contribution of COLIA1 polymorphism to the risk of any fracture and hip fracture, accounting for predefined confounders such as age, BMD, BMI, and history of fractures. For participants who sustained a fracture, the follow-up time was calculated from the date of study entry to the date of the low-trauma fracture (for analyses of any fracture) or a hip fracture (for hip fracture-specific analyses). For those without a fracture, the follow-up time was calculated as the interval between study entry and the date of death or the study end (05/03/2018), whichever came first. The proportional hazards assumption was graphically assessed using Schoenfeld's residuals.

A predefined sensitivity analysis redefined the COLIA 1 polymorphisms as a dominant genotype (*GG/GT* genotypes) versus a recessive genotype (*TT* genotype) to assess any potential improvements in the analytical approach [266]. All statistical analyses were conducted using the R language version 3.6.1 [267].

5.3 Results

5.3.1 Participant characteristics

The study included 809 menopausal women who had both COLIA1 gene data and at least three BMD measurements available (Figure 3.1). Their average age at recruitment was 70 ± 7 years old (Table 3.1). The distribution of genotype frequencies of the *COLIA1* polymorphisms was consistent by the Hardy-Weinberg equilibrium with *GG* (N= 522; 64.5%), *GT* (N= 256; 31.7%), and *TT* (N= 31; 3.8%). There was no significant difference in age, height, weight, BMI, and femoral neck BMD between *COLIA1* genotypes (Table 5.1).

Table 5.1. Baseline clinical characteristics of participants stratified by *COLIA1* genotype

	GG	***GT***	***TT***	**P-value**
No. of patients	522	256	31	
Age (year)	70 (6.5)	70 (6.5)	69 (6.2)	0.41
Height (cm)	160 (6)	161 (6)	162 (7)	0.06
Weight (kg)	66 (11.7)	65 (11.5)	65 (13.7)	0.72
BMI (kg/m^2)	26 (4.5)	25 (4.3)	25 (4.5)	0.15
Femoral neck BMD (g/cm^2)	0.79 (0.13)	0.79 (0.13)	0.78 (0.11)	0.69
History of fractures n(%)	52 (10%)	24 (9.4%)	2 (6.4%)	0.90

Data are mean (SD), otherwise indicated.

5.3.2 Association between *COLIA1* polymorphisms and bone loss

We found that post-menopausal women with the TT genotype had an average rate of bone loss of 0.57% ± 0.43% per year. In comparison, women with the GG and GT genotypes had similar rates of bone loss, at 0.55% ± 0.41% and 0.54% ± 0.41% per year, respectively. These differences were minimal and not statistically significant (P = 0.93), indicating that COLIA1 genotype did not substantially impact the rate of bone loss in this cohort.

5.3.3 Association between COLIA1 polymorphisms and fracture

During a median follow-up of 13 years (IQR 6.6-21.1), 423 (52.3%) women sustained at least one incident low-trauma fracture (Table 5.2). Women who experienced a fracture were older and had lower weight and BMD than those without a fracture. Importantly, we found that postmenopausal women with the *TT* allele had a significantly higher incidence of any fracture (75 fractures/1000 person-years; 95% CI, 48-110) compared to both *GG* (36 fractures/1000 person-years; 95% CI, 32-41) and *GT* carriers (39 fractures/1000 person-years; 95% CI, 33-46) (P = 0.024). During a median follow-up of 19 years (IQR 12-24.2), 120 women experienced a hip fracture, yielding an incidence rate of 8 hip fractures/1000 person-years (95% CI, 7-10). The *TT* carriers had the highest incidence of hip fractures (20 hip fractures/1000 person-years; 95% CI, 9-38), significantly higher than *GG* (7 hip

fractures/1000 person-years; 95% CI, 6-9) and *GT* carriers (9 hip fractures/1000 person-years; 95% CI, 6-12, P = 0.048). Notably, in the predefined sensitivity analysis, women with a recessive genotype (*TT* allele) had a significantly greater risk of any fracture (Figure 5.3A) and hip fracture (Figure 5.3B) compared to those with a dominant genotype (*GG* and *GT* alleles).

Table 5.2. Baseline clinical and anthropometric characteristics of participants stratified by fracture status

	Nonfracture	**Fracture**	**P value**
No. of patients	386	423	
Age (years)	69 (6.3)	70.5 (6.7)	**0.001**
Weight (kg)	67 (12)	64.5 (11)	**0.004**
Height (cm)	160 (5.8)	160 (6.3)	0.587
Body mass index (kg/m^2)	26 (4.6)	25.1 (4.2)	**0.005**
Femoral neck BMD (g/cm^2)	0.83 (0.12)	0.75 (0.12)	**<0.001**
History of fractures			0.06[a]
No fracture n(%)	357 (92.5%)	374 (88.4%)	
Fracture n(%)	29 (7.5%)	49 (11.6%)	
COLIA1 genotypes			**0.02[a]**
GG n(%)	261 (67.6%)	261 (61.7%)	
GT n(%)	117 (30.3%)	139 (32.9%)	
TT n(%)	8 (2.07%)	23 (5.44%)	

Data are mean (SD) and p-values estimated from Student's t-test, unless otherwise indicated. [a]Chi square test.

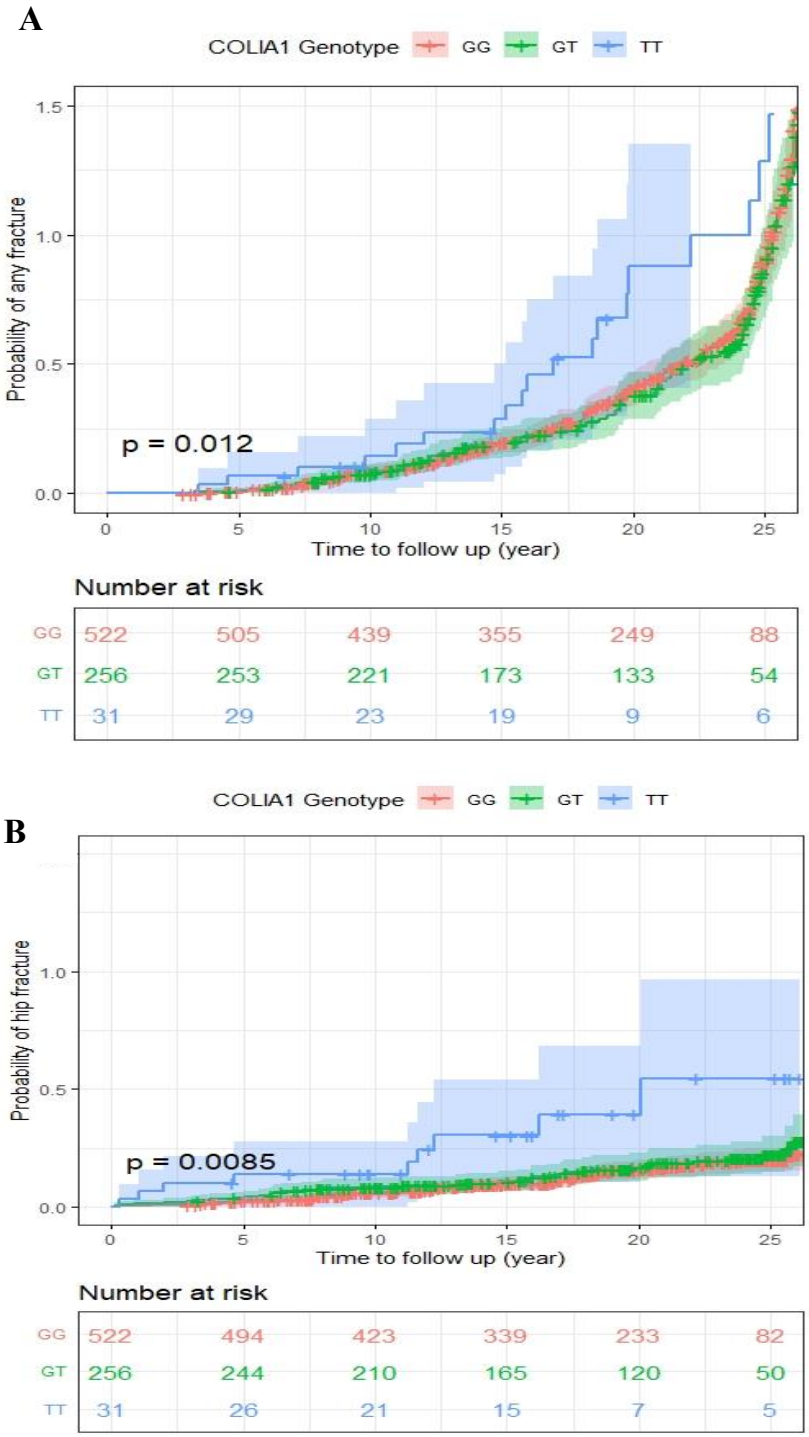

Figure 5.2 Kaplan-Meier curves showing cumulative probability of any fracture (A) and hip fracture (B), stratified by genotype groups (*GG, GT, TT*)

Table 5.3 represents the association between *COLIA1* genotypes and the risk of fractures, adjusted for confounding effects. Women with the *TT* genotype had a 2-fold greater risk of any fracture (HR = 2.20; 95% CI, 1.43-3.38) and a 3.5-fold greater risk of hip fracture (HR = 3.52; 95% CI, 1.75-7.09) than those with the *GG* genotype, after adjusting for differences in age between the genotypes. Importantly, these associations remained significant after further adjustment for baseline BMD, BMI, and history of fractures. By contrast, women with the *GT* genotype were not associated with a significantly greater risk of any or hip fractures compared to *GG* carriers.

A predefined sensitivity analysis was conducted to examine whether collapsing the genotypes would enhance analytical power (Figure 5.3). We found that women with a recessive genotype (*TT*) had a 2-fold and 3-fold greater risk of any fracture and hip fracture, respectively, than those with a dominant genotype (*GG* or *GT*), which was consistent with the primary analysis.

In addition, while the average rate of bone loss was higher among women with fractures (-0.66% per year, SD = 1.63%) compared to those without fractures (-0.48% per year, SD = 1.28%), this difference did not reach statistical significance (P = 0.093). However, when adjusting for other variables in a Cox proportional hazards model, the rate of bone loss was found to be a significant predictor of fracture risk (HR = 0.90; 95% CI, 0.84–0.96; P < 0.001), independent of baseline femoral neck BMD and other confounders. This result indicates that a slower rate of bone loss, or more stable bone density, is associated with a 10% reduction in fracture risk per percentage point change in bone loss rate. Importantly, this association remained significant even after adjusting for baseline BMD, suggesting that the rate of bone loss is an independent risk factor for fractures in this cohort.

Table 5.3 The independent association between COLIA 1 genotypes and fracture risks

Any fracture					
		Age-adjusted		Multivariable adjusted**	
Variables	Number of fractures*	HR (95% CI)	P-value	HR (95% CI)	P-value
GG (N=522)	261 (46.2%)	Reference	-	Reference	-
GT (N=256)	139 (54.3%)	1.06 (0.86-1.30)	0.06	1.05 (0.86-1.30)	0.6
TT (N=31)	23 (74.2%)	**2.20 (1.43- 3.38)**	**<0.001**	**2.17 (1.41-3.34)**	**<0.001**
Hip fracture					
		Age-adjusted		Multivariable adjusted**	
Variables	Number*	HR (95% CI)	P-value	HR (95% CI)	P-value
GG (N=522)	70 (13.4%)	Reference	-	Reference	-
GT (N=256)	41 (16.0%)	1.08 (0.73- 1.59)	0.7	1.03 (0.70- 1.52)	0.9
TT (N=31)	9 (29.0%)	**3.52 (1.75- 7.09)**	**<0.001**	**3.77 (1.86- 7.62)**	**<0.001**

*Data presented as number of patients with a fracture (%). **Hazard ratio for each variable have been adjusted for age, femoral neck BMD, BMI and history of fracture. **Bold font indicates statistical significance.**

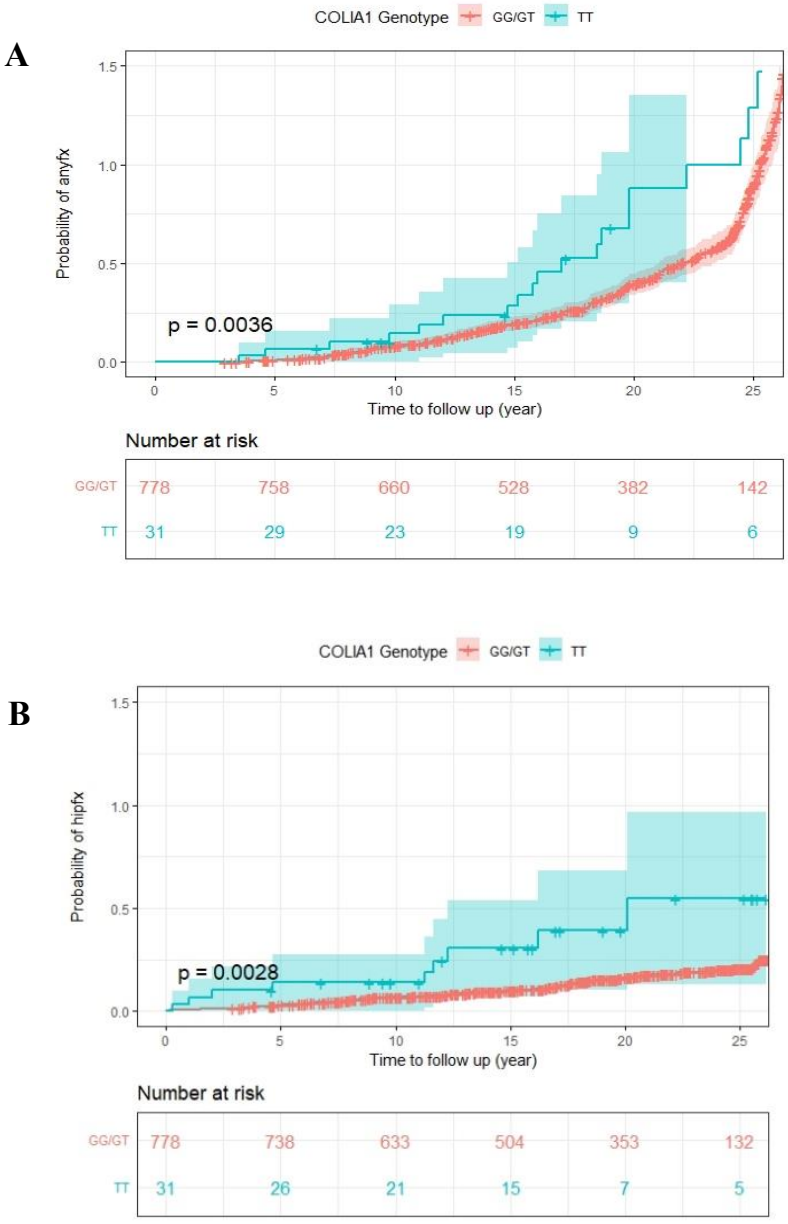

Figure 5.3 Kaplan-Meier curves showing cumulative probability of any fracture (A) and hip fracture (B), stratified into two groups: dominant (*GG + GT)* and recessive (*TT*) of *COLIA1* genotyp

5.4 Discussion

Using a well-established cohort study with biannual measurement of BMD and bone health being monitored for almost 30 years, we found that the *TT* genotype of *COLIA1* gene was associated with a significantly increased risk of any fracture and hip fracture in postmenopausal women. Notably, our study found no significant association between the *COLIA1* gene and BMD or bone loss, suggesting that the association between *COLIA1* and fracture is independent of bone density and bone loss rates. These findings contribute to the understanding of genetic risk factors for fractures, extending the insights from previous research.

Our findings align with earlier studies that reported no significant association between the *COLIA1* gene and BMD [126, 177, 268] or bone loss [269, 270]. In terms of bone loss, Heegaard et al. (2000) examined a Danish cohort followed for 18 years, while McGuigan et al. (2001) analyzed a mixed-gender cohort over a mean follow-up of approximately 5 years [269, 270]. However, our study contributes new insights by examining the relationship between COLIA1 polymorphisms (*TT, GG, GT* genotypes) and both hip fractures and overall fracture risk in a well-characterized cohort of Australian postmenopausal women with a median follow-up of 13 years. Additionally, our study used linear mixed-effects models to more accurately quantify changes in bone density over time, an approach that allows for a more detailed understanding of the dynamics of bone loss compared to conventional methods used in earlier studies. This methodological strength, combined with our focus on fracture outcomes, provides a broader perspective on the role of *COLIA1* in bone health. Unlike previous studies, our analysis includes a specific emphasis on hip fractures, which are particularly debilitating in the elderly population and were not the primary focus in earlier research. While the lack of a significant association between COLIA1 polymorphisms and bone loss may seem consistent with prior findings, our study reinforces the importance of replicating genetic associations across different populations. Such replication is essential for verifying the generalizability of findings and understanding the variable influence of genetic factors like *COLIA1* across diverse groups.

Additionally, our findings align with the previously documented association between the *COLIA1* gene and fragility fractures in postmenopausal women, even after accounting for potential confounding factors [126, 177, 268]. By contrast, the association between COLIA1 gene and BMD was modified by age groups in a study of 1778 postmenopausal women with an average age of 66 ± 7 [125]. Specifically, the significant changes in BMD at femoral neck were only documented in women older than 70 years with minor allele (the average change in BMD per year: -0.031 ± 0.012 g/cm^2, P=0.01), but not in the younger group aged 65-69 years (-0.017± 0.012 g/cm^2, P=0.18). On the other hand, almost 60% of our participants aged 70 years old and younger, possibly explaining differences between our findings and theirs [125].

Importantly, we found that *COLIA1* polymorphisms were associated with fractures independent of BMD and bone loss, a finding consistent with other studies[126, 266, 271]. Our findings reveal a strong correlation between the *TT* genotype and fractures, independent of BMD. This suggests that the increased fracture risk associated with this genotype may be influenced by mechanisms beyond bone density. As *COLIA1* is just one of many genes contributing to osteoporosis susceptibility, our findings align with previous research suggesting that bone mass is influenced by a complex interplay of multiple genes, each exerting modest effects [272-274]. Furthermore, our documented association between *COLIA1* gene and fracture independent of BMD may be related to bone microarchitecture or quality. Functional studies have shown that individuals with the *TT* genotype exhibit an altered ratio between the produced amount of collagen (type I) chain α1 (*COLIA1*) and the collagen chain α2 (*COLIA2*) [275, 276]. Changes in this ratio could lead to reduced peak bone mass or alterations in bone structure, as observed in conditions such as osteogenesis imperfecta. Individuals with the *TT* genotype, characterized by altered collagen production, may experience a decrease in trabecular thickness, making them more susceptible to trabecular perforations during high turnover periods associated with aging or menopause. Consequently, this could result in a more pronounced reduction in bone strength compared to the concurrent loss of bone density.

The findings of this study must be interpreted in the context of its strengths and weaknesses. The study's strengths include a well-characterized cohort in which the participants' bone health was monitored biannually for almost 30 years. Importantly, BMD was measured using the standard method, and all fractures were radiologically confirmed. A linear mixed-effects regression to quantify bone loss is considered more rigorous than the conventional linear regression, improving the estimation accuracy and enhancing the study's internal validity [277]. Additionally, the predefined sensitivity analysis with the collapsed genotypes produced similar findings, confirming the robustness of the results. However, since the majority of participants in the study are of Caucasian descent, its findings might not be generalisable to other ethnicities.

In summary, our study documents a significant association between *COLIA1* polymorphism and fragility fractures, independent of age, bone mineral density, and bone loss. While our findings align with earlier reports on the lack of association between *COLIA1* and BMD, they extend the understanding of fracture risk by emphasizing the potential role of genetic factors beyond bone density measures. These findings underscore the potential utility of *COLIA1* genotyping in enhancing fracture risk prediction, particularly for individuals with specific genetic profiles. Our study provides a valuable contribution to the ongoing research on genetic determinants of osteoporosis and may inform targeted prevention strategies.

CHAPTER 6.

NOVEL GENETIC VARIANTS ASSOCIATED WITH BONE MINERAL DENSITY IN ASIANS: THE VIETNAM OSTEOPOROSIS STUDY

6. Novel Genetic Variants Associated with Bone Mineral Density in Asians: the Vietnam Osteoporosis Study

6.1 Introduction

Osteoporosis is a chronic condition characterized by reduced bone mineral density (BMD) and deterioration of bone microarchitecture, which leads to increased bone fragility and a heightened risk of fractures. BMD is widely recognized as the most significant diagnostic indicator for osteoporotic fractures [278, 279]. While environmental factors influence the development and progression of osteoporosis, genetic factors play a pivotal role, as evidenced by the high heritability of BMD in both genders [280]. Heritability estimates for BMD are particularly high, with studies estimating heritability at 78% at the lumbar spine and 84% for the femoral neck [281].

In previous chapters, we examined the role of genetic factors, particularly the COLIA1 gene, in osteoporosis susceptibility and fracture risk independent of BMD. However, these findings underscore the need to further explore other genetic variants, particularly those influencing bone loss, to better understand their contributions to osteoporosis and fracture risk. Genome-wide association studies (GWAS) have been instrumental in identifying loci related to BMD and fracture susceptibility [282-285]. However, research specifically addressing bone loss is limited due to the complex requirement of multiple BMD measurements to accurately assess changes over time. This gap is particularly evident in non-European populations, where GWAS on BMD are relatively sparse [284, 286, 287]. Our study addresses this limitation by focusing on the Vietnamese population, which has been underrepresented in genetic studies of BMD.

Due to the limited availability of multiple BMD measurements in the Vietnam Osteoporosis Study (VOS) dataset, this study focuses on establishing a foundational understanding of genetic influences on BMD in a Vietnamese cohort rather than evaluating bone loss as originally intended. To our knowledge, this represents the first GWAS of BMD

in the Vietnamese population, providing essential insights into population-specific genetic variants.

This study uses data from the Vietnam Osteoporosis Study to investigate genetic variants influencing BMD at critical skeletal sites—the lumbar spine, femoral neck, and total hip—through GWAS. Additionally, we employ Polygenic Risk Score (PRS) analysis, which offers a numerical estimate of an individual's genetic predisposition to a specific trait or disorder. PRS is calculated by summing the effects of multiple genetic variants across the genome, typically weighted by their effect sizes derived from GWAS [19, 288]. PRS analysis will further enable the exploration of genetic correlations among complex traits related to BMD [289-291].

This study's significance lies in its focus on an underrepresented population and its use of PRS to enhance the understanding of genetic factors influencing BMD. The finding will provide an essential foundation for understanding genetic influences on bone health in the Vietnamese population and establishes a groundwork for future research on bone loss and fracture susceptibility in diverse populations. This chapter aims to uncover loci linked to BMD at key skeletal sites, including the lumbar spine, femoral neck, and total hip.

6.2. Study design and method

Data for this study were obtained from the Vietnam Osteoporosis Study (VOS), as described in chapter 2.

6.2.1 GWAS analysis

Single nucleotide polymorphisms (SNPs) and samples were filtered based on the quality criteria: Hardy-Weinberg equilibrium $p < 1 \times 10^{-4}$, a call rate < 98%, and minor allele frequency < 1%, using PLINK software package version 1.9 [292].

Genome-wide Complex Trait Analysis (GCTA) software was employed to quantify the influence of common genetic variants on three phenotypes of BMD and to investigate the shared genetic basis of these trait [293, 294]. GCTA estimates the heritability attributable to

SNPs and the genetic correlation by analyzing genome-wide similarities between distantly related individuals. A genetic relationship matrix (GRM), capturing the genetic relatedness among individuals, was used as input for the GCTA analysis. Participants were excluded to ensure that no pair exceeded the standard GCTA cut-off coefficient of 0.05 for genetic relatedness. A mixed linear model was performed in GCTA, including age and BMI as continuous covariates and sex as a categorical covariate. After quality control, genotype data for 386,455 autosomal variants were included for further analysis (Figure 6.1).

Quantile-quantile (Q-Q) and Manhattan plots were generated in R Statistical Environment using the qqman package [224, 295]. Regional association plots of the significant SNPs were generated by LocusZoom software [296]. The genome-wide level of statistical significance was set at $P < 1 \times 10^{-7}$, and the suggestive level of significance was set at $P < 5 \times 10^{-6}$ to identify potentially meaningful associations that might be overlooked [297, 298]. The identification of regulatory SNPs was accomplished using ENCODE data [299], as documented in RegulomeDB [300].

6.2.2 Polygenic risk scores analysis

Polygenic Risk Scores (PRS) were computed using GWAS summary statistics and the Bayesian polygenic prediction method, PRS-CS, following several quality control steps. Initially, kinship analysis was performed to retain participants with a kinship coefficient ≤ 0.125, resulting in a final sample of 1,618 participants (591 males and 1,027 females). Subsequently, linkage disequilibrium (LD) pruning was applied with an r^2 threshold of 0.1 and a 250 kb window. PRS computation was carried out using PLINK software (version 1.9), where the effects of the clumped SNPs were weighted by their effect sizes derived from GWAS summary statistics. Statistical analysis of the PRS was then performed using R programming. Various *P*-value thresholds were applied to identify significant SNPs for PRS calculation. After calculating the PRS for each threshold, the genetic contributions to BMD were assessed by evaluating the R-squared change in linear models that included the PRS scores as a covariate.

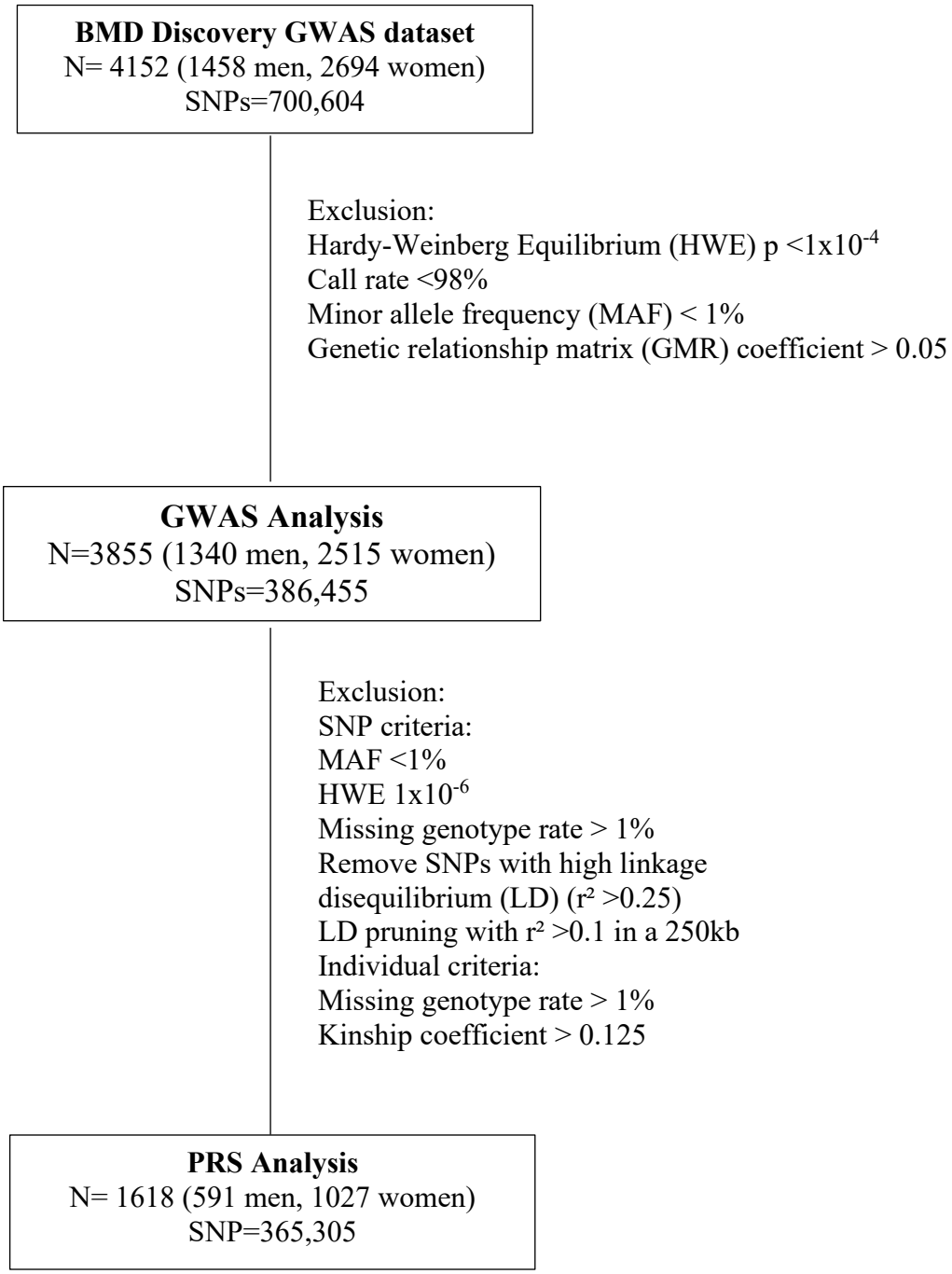

Figure 6.1 Flowchart for analysis of genome-wide association study (GWAS) and Polygenic risk score (PRS) data for bone mineral density (BMD)

6.3 Results

Our study was restricted to Vietnamese participants to minimize population stratification, resulting in a cohort of 4,152 individuals with genotype and phenotype data (Table 6.1). The mean age of the participants was 49 years in women and 46 years in men, with BMI between 23 kg/m^2 in both sexes. We examined distribution patterns of three BMD phenotypes: femur beck BMD (FNBMD), lumbar spine BMD (LSBMD), and total hip BMD (HTOTBMD). LSBMD ranged between 0.37-1.59 g/cm^2 (mean ± SD, 0.88 ± 0.15 g/cm^2) in women, and 0.54-1.50 g/cm^2 (mean ± SD, 0.95 ± 0.13 g/cm^2) in men. FNBMD ranged between 0.23-1.20 g/cm^2 (mean ± SD, 0.68 ± 0.13 g/cm^2) in women, and 0.41-2.64 g/cm^2 (mean ± SD, 0.79 ± 0.15 g/cm^2) in men. HTOTBMD ranged between 0.32-1.39 g/cm^2 (mean ± SD, 0.80 ± 0.12 g/cm^2) in women, and 0.41-2.40 g/cm^2 (mean ± SD, 0.90 ± 0.14 g/cm^2) in men (Table 6.1).

Table 6.1 Baseline characteristics of VOS

Baseline characteristics	**Women N = 2694**	**Men N = 1458**	**P-value**
Age (yrs)	49 (14)	46 (15)	<0.001
Weight (kg)	53 (8.2)	63 (10.1)	<0.001
Height (cm)	153 (5.7)	164 (6.6)	0.000
BMI (kg/m^2)	22.8 (3.3)	23.3 (3.3)	<0.001
Femoral neck BMD (g/cm^2)	0.68 (0.13)	0.79 (0.15)	<0.001
Lumbar spine BMD (g/cm^2)	0.88 (0.15)	0.95 (0.13)	<0.001
Total hip BMD (g/cm^2)	0.80 (0.12)	0.90 (0.14)	<0.001
Cigarette Smoking			<0.001
No	2670 (99%)	858 (59%)	
Yes	24 (1%)	600 (41%)	
Alcohol intake			<0.001
No	2612 (97%)	805 (55%)	
Yes	82 (3 %)	653 (45%)	

Values are presented as mean (SD) unless otherwise specified.

As described in the Methods section, we applied quality control measures to the initial set of 700,004 SNPs. First, we filtered for minor allele frequency (MAF) greater than 0.01, yielding 387,323 autosomal SNPs. Subsequently, we used a genetic relationship matrix (GRM) to exclude individuals with genetic relatedness exceeding 0.05. These steps resulted in a final set of 386,455 SNPs for subsequent analyses (Figure 6.1).

We identified seven novel SNPs with a significant influence on BMD (Table 6.2). Rs4689808 ($P = 2.9 \times 10^{-6}$), and rs7186410 ($P = 6.9 \times 10^{-7}$) were significantly associated with LSBMD, and rs11066695 ($P = 4.4 \times 10^{-6}$), rs7325467 ($P = 2.7 \times 10^{-7}$), rs528202723 ($P = 4.2 \times 10^{-7}$), rs201921260 ($P = 1.4 \times 10^{-11}$), and rs766843 ($P = 3.4 \times 10^{-7}$) were significantly associated with FNBMD (Table 6.2). One SNPs, rs201921260 were significantly associated with HTOTBMD ($P = 9.0 \times 10^{-10}$), which were previously found in FNBMD ($P = 1.4 \times 10^{-11}$).

Figures 6.2, 6.3, and 6.4 illustrate regional plots of the seven significant SNPs within 400 kilobases in LSBMD, FNBMD, and HTOTBMD, respectively. Quantile–quantile and Manhattan plots of the P-values are shown in Supplementary Figures. 6.1 and 6.2. Examination of genomic location revealed that rs4689808 is located in the sortilin-related VPS10 domain containing receptor 2 (SORCS2) gene at 4p16.1 (Figure 6.2A). Rs7186410 is in a long intergenic non-protein coding RNA 2131 (LINC02131) locus at 16q23.1 (Figure 6.2B). While these two SNPs were associated with LSBMD, five other SNPs were linked to FNBMD.

Rs528202723 is in the protein-coding of ATXN10 gene located in 22q13.31 (Figure 6.3A), whereas rs11066695 at 12q24.13 is currently of undetermined function (Figure 6.3C). Rs7325467 is located in a non-coding RNA region at 13q12.12 (Figure 6.3B), while rs766843 is a single nucleotide variation at locus Xq25 (Figure 6.3D). The functional implications of these latter two SNPs are also currently undetermined. Among these seven SNPs, rs201921260 was the only novel SNP associated with both FNBMD and HTOTBMD. It was a missense variant of G protein-coupled receptor associated sorting protein 1 (GPRASP1) **(**Figure 6.4).

Table 6.2 Summary of SNPs significantly associated with bone mineral density at lumbar spine, femoral neck, and total hip

No	Lous	SNP (rs ID)	Closest gene	Genetic function	Gwas catalog (Trait: BMD) Novel or reported SNPs	β-value	P-value[d]
LSBMD[a]							
1	4p16.1	rs4689808	SORCS2	Intronic	Novel	0.0159	**2.9x10e-6**
2	6q25.1	rs13213582	CCDC170	Intronic	Reported	0.0169	9.7x10e-8
3	6q25.1	rs1871859	CCDC170	Intronic	Reported	0.0196	8.6x10e-10
4	16q23.1	rs7186410	NUDT7	Upstream	Novel	0.0180	**6.9x10e-7**
FNBMD[b]							
5	7q31.31	rs3779381	WNT16	Intronic	Reported	0.024	9.8x10e-9
6	7q31.31	rs2908004	WNT16	Missense variant	Reported	0.021	2.4x10e-9
7	7q31.31	rs3801387	WNT16	Intronic	Reported	0.024	7.7 x10e-9
8	7q31.31	rs2707466	WNT16	Missense variant	Reported	0.020	1.2 x10e-8
9	7q31.31	rs10242100	WNT16	Downstream	Reported	0.022	9.4 x10e-8
10	7q31.31	rs917727	FAM3C	Intronic	Reported	0.022	3.8 x10e-8
11	7q31.31	rs7776725	FAM3C	Intronic	Reported	0.024	2.2 x10e-8
12	12q24.13	rs11066695	LINC01234	Upstream	Novel	0.026	**4.4x10e-6**
13	13q12.12	rs7325467	HMGA1P6	Upstream	Novel	0.021	**2.7 x10e-7**
14	22q13.31	rs528202723	ATXN10	Intronic	Novel	0.093	**4.2 x10e-7**
15	Xq22.1	rs201921260	GPRASP1	Missense variant	Novel	0.096	1.4 x10e-11
16	Xq25	rs766843	MRRFP1	Upstream	Novel	0.026	**3.4 x10e-7**
HTOTBMD[c]							
17	7q31.31	rs3779381*	WNT16	Intronic	Reported	0.022	4.6 x10e-8
18	7q31.31	rs2908004*	WNT16	Missense variant	Reported	0.020	7.9 x10e-9
19	7q31.31	rs3801387*	WNT16	Intronic	Reported	0.023	3.4 x10e-8
20	7q31.31	rs2707466*	WNT16	Missense variant	Reported	0.019	2.1 x10e-8
21	7q31.31	rs10242100*	WNT16	Downstream	Reported	0.021	**2.8 x10e-7**
22	7q31.31	rs917727*	FAM3C	Intronic	Reported	0.021	**1.3 x10e-7**

23	7q31.31	rs7776725*	FAM3C	Intronic	Reported	0.023	4.1 x10e-8
24	Xq22.1	rs201921260*	GPRASP1	Missense variant	Novel	0.085	9.0 x10e-10

SNP, single nucleotide polymorphism; SORCS2, sortilin related VPS10 domain containing receptor 2; CCDC170, coiled-coil domain containing 170; LINC02131, long intergenic non-protein coding RNA 2131; WNT16, Wnt family member 16; FAM3C, family with sequence similarity 3, member C; LINC01234, long intergenic non-protein coding RNA 1234; HMGA1P6, high mobility group AT-hook 1 pseudogene 6; ATXN10, Ataxin 10; GPRASP1, G protein-coupled receptor associated sorting protein 1; MRRFP1, mitochondrial ribosome recycling factor pseudogene 1.

P-values smaller than the genome-wide significance threshold ($P < 1 \times 10^{-7}$) are reported, with those at the suggestive significance threshold ($P < 5 \times 10^{-6}$) indicated in bold.

*SNPs were significantly associated with both FNBMD and HTOTBMD.

[a] Bone mineral density at lumbar spine; [b] Bone mineral density of femur neck; [c] Bone mineral density of total hip;

[d] The *P*-values are based on a mixed-effect model adjusted for age, sex, and BMI

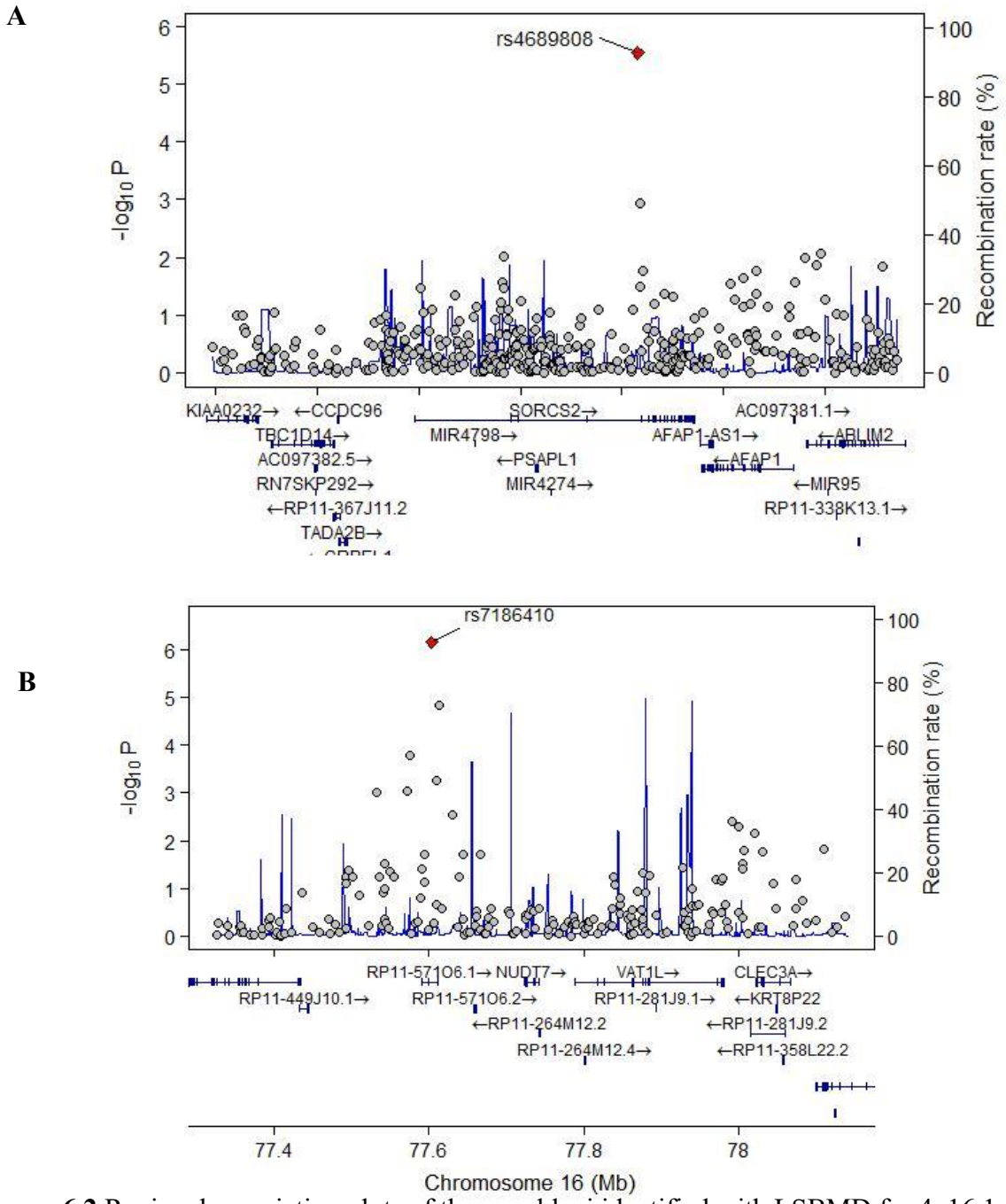

Figure 6.2 Regional association plots of the novel loci identified with LSBMD for 4p16.1 (**A**) and 16q23.1 (**B**). Genetic variants within 400 kb of the lead variant are depicted (x-axis) along with their meta-analysis P value ($-\log_{10}$). The positions of genes and direction of transcription are also indicated.

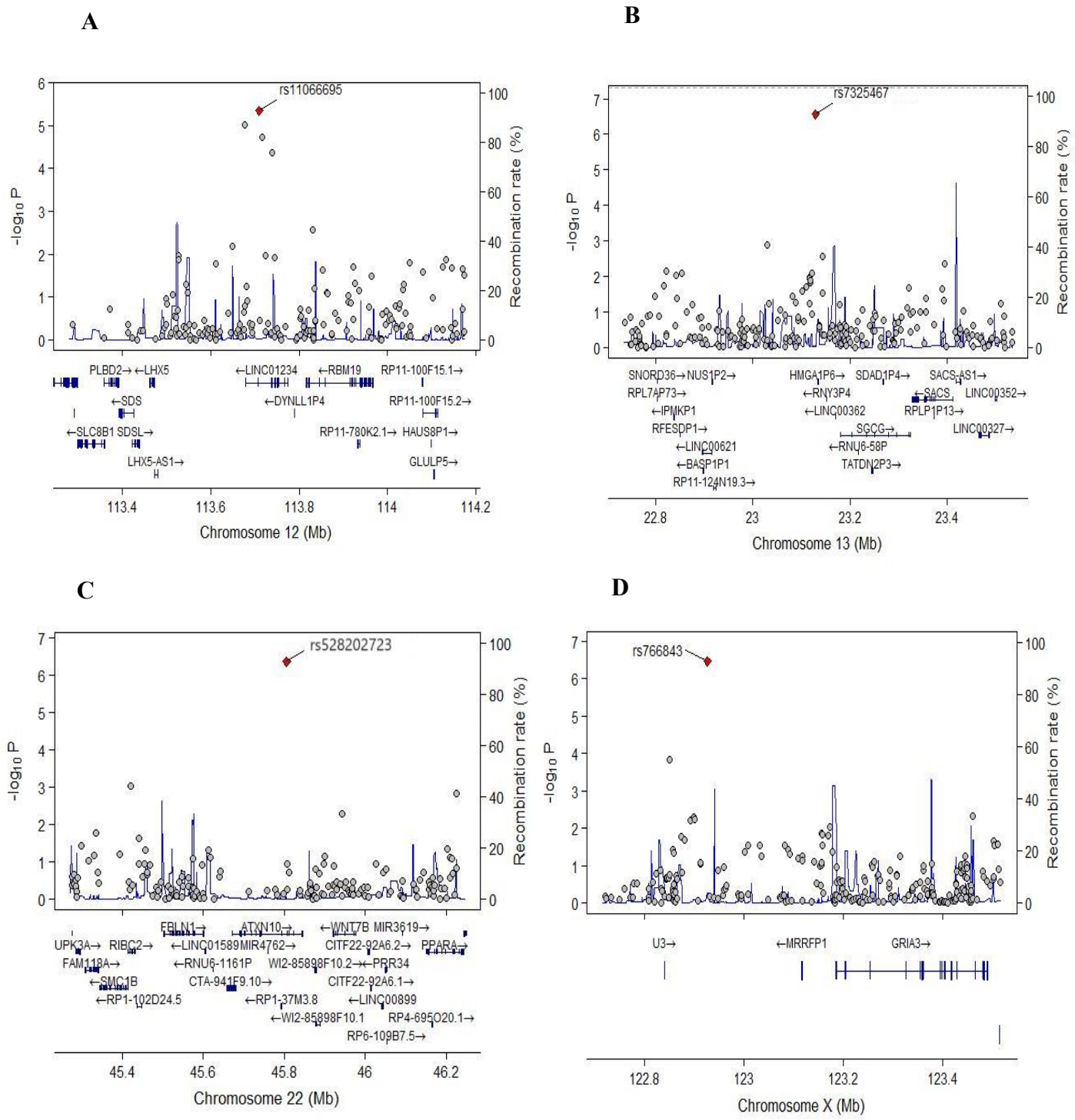

Figure 6.3 Regional association plots of the novel loci identified with FNBMD for 12q24.13 (**A**) 13q12.12 (**B**) 22q13.31 (**C**) Xq25(**D**). Genetic variants within 400 kb of the lead variant are depicted (x-axis) along with their meta-analysis P value ($-\log_{10}$). The positions of genes and direction of transcription are also indicated.

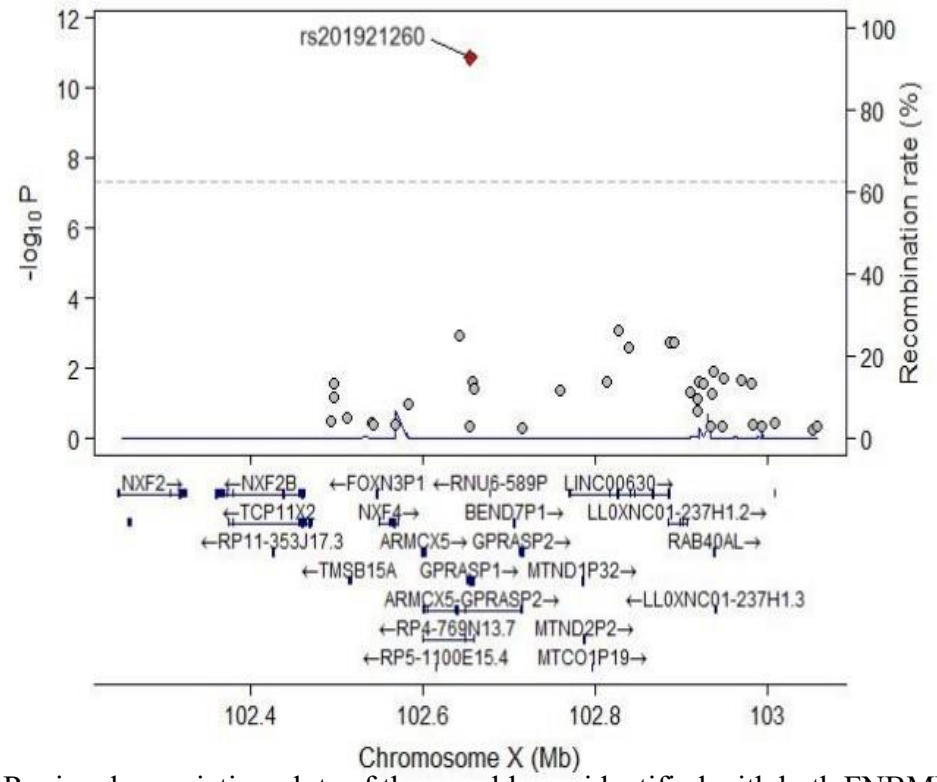

Figure 6.4. Regional association plots of the novel locus identified with both FNBMD and LSBMD for Xq22.1. Genetic variants within 400 kb of the lead variant are depicted (x-axis) along with their meta-analysis P value ($-\log_{10}$). The position of gene and direction of transcription are also indicated.

In addition to establishing links between genetic polymorphisms and BMD, our studies identified nine loci previously confirmed as osteoporosis factors in other population verifying our outcomes (Table 6.2). Rs3779381 (P =9.8x10^{-9}), rs2908004 (P =2.4x10^{-9}), rs3801387 (P =7.7 x10^{-9}), rs2707466 (P =1.2 x10^{-8}), and rs10242100 (P =9.4 x10^{-8}), all in WNT16 were associated with FNBMD. Rs917727 and rs7776725 in FAM3C also exhibited a high association with FNBND, with P = 3.8x10^{-8} and P=2.2x10^{-8}, respectively. Rs10242100 (P =9.4 x10^{-8}) was also linked to femoral neck region, albeit with high p-value that was in both WNT16 and FAM3C. Patterns of rs13213582 (P =9.7x10^{-8}) and rs1871859 (P =8.6x10^{-10}) in CCDC170 were associated with LSBMD. All those previous confirmed SNPs that were associated with FNBMD and LSBMD, were found to influence HTOTBMD, albeit with high p-value (P <5x10^{-6}).

The polygenic risk scores (PRS) in this study were calculated using PRS-CS and tested across various P-value thresholds: 0.001, 0.005, 1x 10^{-4}, 5x10^{-4}, 1x10^{-5}. The best-fitting PRS was identified at a threshold of 1x 10^{-4}, which was based on a sample of 1,618 individuals. The best-fit PRS explained 1.8% of the variance in lumbar spine bone mineral density (LSBMD), 0.73% in femoral neck bone mineral density (FNBMD), and 0.82% in total hip bone mineral density (HTOTBMD). Whereas PRS based on our P-value threshold of 5x10^{-6} explained less than 1.3% of the variance in LSBMD, less than 0.67% in FNBMD, and less than 0.56% in HTOTBMD (Figure S6.3).

6.4 Discussion

This genetic association study was conducted for BMD using a Vietnamese cohort of 4,152 individuals. We identified 384,455 SNPs with genome-wide significant associations to BMD at the lumbar spine, femoral neck, and total hip. These were refined to 365,305 SNPs from 1,618 individuals for the PRS analysis.

It has been shown that limiting predictor SNPs to those with genome-wide significance can be overly restrictive when generating genetic risk scores for highly polygenic traits [301, 302]. In our study, PRS was calculated using a less stringent threshold of 1x 10^{-4}, as many SNPs with less stringent P-values still provide valuable information. Consistent with previous results

[162, 303, 304], this study demonstrates that part of the genetic basis for the BMD phenotype is polygenic, with many common variants contributing small effects. We observed limited correlations between individual SNPs and BMD, with BMD value adjusted for age, sex, and BMI

Our study replicated 50 out of 52 significant SNPs identified in the genetic association study by Estrada et al. for FNBMD and LSBMD [162]. However, only 13 out of the 78 significant SNPs identified by Lin et al. in the Taiwan Biobank study were replicated [305], and none of the 11 significant SNPs identified in a Korean study were replicated [306]. These disparities suggest that unreplicated SNPs may be false positives or might reflect differences in anatomical sites or populations studied.

We identified seven novel SNPs associated with BMD in the Vietnamese population. Two SNPs (rs4689808 and rs7186410) were linked with LSBMD. Four SNPs (rs11066695, rs7325467, rs528202723, and rs766843) were associated with FNBMD, and one SNP (rs2019212600) was connected to both HTOTBMD and FNBMD.

We confirmed nine SNPs previously established in genetic studies in the Vietnamese population. LSBMD was connected to two loci (rs13213582 and rs1871859) in CCDC170, while FNBMD and HTOTBMD were associated with loci in WNT16 (rs3779381, rs2908004, rs3801387, rs2707466, and rs10242100), and FAM3C (rs917727 and rs7776725).

Rs4689808 is located on 4p16.1, within the Sortilin Related VPS10 Domain containing receptor 2 (SORCS2) gene. SORCS2 is a member of the vacuolar protein sorting 10 (VPS10) domain-containing receptor proteins. A previous genome-wide association study found a locus of the SORCS2 gene to be associated with insulin-like growth factor-binding protein-3 (IGFBP-3), a protein involved in bone metabolism [307]. Although the relationship between this gene and BMD is not clear, it is possible that the expression of SORCS2 could affect bone mineral density formation through methylation [308].

Both rs7186410 and rs11066695 are located within long non-coding RNA (lncRNA). LncRNAs are RNAs longer than 200 nucleotides that do not encode proteins but play roles in

various cellular processes, including gene expression regulation [309]. Rs7186410 is found on 16q23.1 within LINC02131. While its specific functions of LINC02131 are not fully understood, it may indirectly affect BMD by influencing the expression of genes involved in bone metabolism. Further research is needed to elucidate its potential impact on bone health.

In contrast, rs11066695 is located on 12q24.13 within the LINC01234, which has been directly implicated in osteoporosis progression [310, 311]. LINC01234 is highly expressed in the plasma of osteoporosis patients and inhibits osteogenic differentiation of human mesenchymal stem cells (hMSCs) by upregulating aldehyde oxidase 1 (AOX1) via miR-513a-5p suppression [310]. AOX1 inhibits osteogenesis through retinoid X receptor (RXR) signalling, as evidenced by RXR receptor-targeting ligands replicating AOX1's effects on bone formation [312]. These findings suggest that targeting LINC01234 could potentially enhance osteogenesis and impede osteoporosis development.

Rs7325467 is located on 13q12.12 within an uncharacterized RNA gene (LOC105370112). It is upstream of the high mobility group AT-hook 1 pseudogene 6 (HMGA1P6), which acts as a competitive endogenous RNA (ceRNA). CeRNAs can enhance the expression of their target genes, in this case, HMGA1 and HMGA2 [313]. Previous studies suggest HMGA2 is involved in bone development through its role in bone homeostasis [314-316]. HMGA2 plays a critical role in regulating bone marrow-derived mesenchymal stem cell (MSC) differentiation into osteoblasts and negatively regulates osteoblast genesis [317]. SNPs located upstream HMGA1P6 may influence HMGA2’s expression, potentially contributing to BMD variations through effects on osteoblast differentiation and osteocyte homeostasis. However, further research is needed to elucidate the specific impact of rs7325467 on bone health.

Rs201921260 is located in the G protein-coupled receptor-associated sorting protein 1 (GPRASP1) gene, which plays a role in the regulation and trafficking of G protein-coupled receptors (GPCRs)[318]. Although there is no clear evidence linking this gene directly to bone health, several GPCRs are known to play roles in bone formation and resorption, influencing osteoblast and osteoclast activity, which are crucial for maintaining bone mineral density [319].

It is possible that GPRASP1 could have indirect effects on BMD through its regulation of GPCRs, but further investigation of this research is imperative.

Rs528202723 is located in the Ataxin-10 (ATXN10) gene, which encodes a protein associated with spinocerebellar ataxia type 10 (SCA10), a rare autosomal-dominant progressive cerebellar syndrome [320]. However, evidence connecting ATXN10 to bone health remains insufficient. Similarly, rs766843 is located on Xq25 and is upstream of a pseudogene gene namely MRRFP1. Currently, there is no scientific evidence establishing a role for these genes in BMD development and maintenance.

While this study was conducted on a large, well-characterized cohort, it is the first to report the significance of these seven novel SNPs in relation to BMD. No replication studies were performed, and the underlying mechanisms through which these SNPs influence BMD remain unknown. Additionally, we did not evaluate other risk factors for osteoporosis, such as family history of the condition, nutritional status, and physical activity levels. Future studies should explore the interplay between these genetic and non-genetic factors to gain a comprehensive understanding of their impact on bone mineral density and osteoporosis.

In conclusion, this GWAS analysis identified seven novel loci associated with BMD in the Vietnamese population, explaining an additional 0.56% to 1.3% of the heritability for BMD. These findings provide valuable insights into the shared genetic factors influencing BMD in Southeast Asians and highlight the polygenic nature of this complex trait. Further research on these novel SNPs can enhance our understanding of osteoporosis pathophysiology across diverse populations.

CHAPTER 7.

SUMMARY AND FUTURE DIRECTIONS

7. Summary and Future Directions

7.1 Summary

Osteoporosis is a major age-related disease with increasing prevalence worldwide due to population aging. Its impact is particularly severe in individuals over 50, leading to fractures, increased disability, and rising healthcare costs [321]. Given that one in four men and two in five women over 50 in Australia will experience a minimal trauma fracture in their remaining lifetime [322], effective prevention strategies are essential. This thesis examined genetic contributions to osteoporosis and bone loss, with additional assessment of lifestyle influences such as smoking, alcohol consumption, and dietary factors.

Together, these findings emphasize the multifactorial nature of osteoporosis, with both genetic and lifestyle factors contributing to bone loss and fracture risk. This thesis advances our understanding of how these influences interact and highlights the need for personalized strategies in osteoporosis management.

7.1.1 *Genetic factors play a crucial role in determining susceptibility to osteoporosis*.

The COLIA1 gene, which is essential for bone structure formation, has been extensively studied for its potential role in osteoporosis. However, its impact remains controversial. While some studies have shown associations between COLIA1 polymorphisms and reduced bone mineral density (BMD) with higher fracture susceptibility, particularly in older women, others have found no significant relationship between COLIA1 and BMD or bone loss.

Our research contributes to this ongoing debate by demonstrating that COLIA1 polymorphisms are significantly associated with increased fracture risk, independent of BMD and bone loss. This finding suggests that genetic factors beyond bone mass, potentially linked to bone quality or microarchitecture, play a crucial role in fracture susceptibility. These insights into the complex relationship between genetics and osteoporosis—particularly how COLIA1's influence on fracture risk extends beyond bone density alone—could refine genetic screening strategies and lead to more personalized approaches in osteoporosis prevention and treatment.

7.1.2 *Lifestyle factors were important determinants of bone loss among the older.*

Understanding lifestyle determinants of bone loss is essential for developing effective prevention strategies for osteoporosis. While factors such as dietary calcium intake and physical activity are known to influence bone mineral density (BMD), their effects on longitudinal bone loss have been less consistent.

In this study, we analyzed data from large, well-characterized cohorts (SOF and MrOS) with extended follow-up periods. Our findings showed that cigarette smoking was associated with accelerated femoral neck BMD loss in women but not in men, while moderate alcohol consumption appeared to slow bone loss in both sexes. Higher physical activity was linked to reduced bone loss at the femoral neck in men, but no significant association was observed in women. Additionally, higher total calcium intake was associated with reduced bone loss in women, whereas dietary protein intake showed no significant effect in either sex.

Although these lifestyle factors were statistically significant, they collectively explained less than 4.2% of the variance in bone loss, highlighting the complexity and suggesting that other biological or genetic mechanisms are likely involved.

7.1.3 *Higher rates of bone loss have been linked to an increased risk of mortality in both men and women*.

As bone loss accelerates with advancing age rather than stabilizing or slowing (as shown in Chapter 4), understanding its relationship with mortality becomes increasingly important. Our study demonstrates that both rapid bone loss and significant bone changes are independently associated with increased mortality risk in older men and women, regardless of age, initial BMD, BMI, smoking status, or drinking status.

Using multiple BMD assessments, we found that individuals experiencing rapid bone loss had a higher mortality risk compared to those maintaining stable BMD. This association persisted after adjusting for baseline BMD and other health-related factors, suggesting that bone loss serves as a critical health indicator distinct from static BMD measurements. These findings

highlight the importance of monitoring bone density changes over time and support bone loss as an independent risk factor for adverse mortality outcomes.

It is important to note that confounders such as BMI, smoking status, alcohol consumption, medication use, and comorbidities were measured at baseline and not updated during follow-up, which may affect the precision of their estimated influence on the bone loss–mortality relationship.

7.1.4 *The genetic basis for BMD phenotype is polygenic*.

The genetic basis for bone mineral density (BMD) is complex and highly polygenic. Using genome-wide association studies (GWAS) in a Vietnamese cohort, we identified novel single nucleotide polymorphisms (SNPs) related to BMD, representing the first such analysis in Vietnam. To explore the cumulative contribution of these variants, we conducted Polygenic Risk Score (PRS) analysis.

Interestingly, contrary to expectations, including more SNPs by decreasing the P-value threshold did not significantly increase the explained variance. The best-fit PRS at a P-value threshold of $1x10^{-4}$ explained 1.8% of the variance in lumbar spine BMD, while a more stringent threshold of $5x10^{-6}$ explained less than 1.3%. Similar trends were observed for total hip and femoral neck BMD. These findings demonstrate that multiple SNPs contribute small individual effects to BMD variation, confirming its polygenic nature. This study provides a valuable foundation for future research on osteoporosis genetics in Southeast Asian populations

7.1.5 *The genetic variants (SNPs) associated with BMD can vary significantly across different ethnic background*.

Genetic variants (SNPs) associated with BMD can vary significantly across different ethnic backgrounds. Our study of a Vietnamese cohort demonstrates this variation: 11 SNPs previously associated with BMD in Korean populations were not detected in our cohort, and only 13 out of 78 SNPs significant in Taiwanese populations were replicated in our study. This limited overlap highlights the substantial genetic diversity across Asian populations, even within the same geographical region.

These findings underscore the importance of conducting genome-wide association studies (GWAS) in diverse ethnic groups. Understanding population-specific genetic influences on BMD is crucial for developing a comprehensive picture of osteoporosis genetics and implementing more effective, personalized prevention and treatment strategies tailored to specific populations.

7.2 Future Directions

Historically, osteoporosis studies focused on quantifying disease frequency, identifying vulnerable demographic groups, and developing predictive tools for fracture risk based on clinical factors. Recent research has shifted toward understanding bone loss progression, the role of genetic and lifestyle factors, and their interactions in osteoporosis. This approach examines bone loss progression, identifies influencing factors, and explores the interaction between genetic predispositions and lifestyle factors.

Future research is likely to emphasize precision medicine and personalized risk assessment, focusing on more targeted prevention and comprehensive strategies to maintain bone health in older adults. Key areas for future exploration include:

(i) Development of polygenic risk scores for better risk stratification.
(ii) Evaluation of genomics, proteomics, and metabolomics interactions to uncover new insights into osteoporosis mechanisms.
(iii) Introduction of new tools to improve doctor-patient communication and treatment uptake for osteoporosis.
(iv) Utilization of diverse populations to enhance the accuracy of risk prediction models
(v) Optimization of personalized lifestyle interventions tailored to genetic profiles

7.2.1 Development of polygenic risk scores for better risk stratification

PRS is an important tool for estimating the effects of genetic variants identified in GWAS on bone phenotypes such as bone mineral density and bone loss. Over the past 15 years, various

PRS methodologies have been developed and improved to enhance accuracy in predicting bone loss and osteoporosis. However, limitations in PRS still exist and need to be addressed.

Most PRS models are based on European ancestry data, which can lead to bias and reduced accuracy when applied to other populations due to differences in linkage disequilibrium patterns and allele frequencies. To improve accuracy and enhance generalizability, data from multiple ancestries across populations should be incorporated.

While PRS has been used for BMD or bone loss prediction, the explained variance in these traits remains modest, indicating room for improvement. Additionally, rather than focusing solely on GWAS data for DNA sequencing, PRS could be enhanced by incorporating other omics data related to the biological pathway of bone phenotypes, such as transcriptomics and proteomics. This evaluation of genomics with other omics data could potentially improve PRS accuracy.

7.2.2 Evaluation of genomics, proteomics, and metabolomics interactions to uncover new insights into osteoporosis mechanisms

While GWAS helps identify SNPs associated with BMD, a comprehensive understanding requires examining the complex interactions between genes, proteins, and metabolites. Protein studies shed light on biological functions, while small molecules and metabolites are crucial for understanding pathways and biochemical changes. Integrating these omics data with GWAS can provide a better understanding of the biochemical changes associated with bone health. This approach may lead to the discovery of novel biomarkers for early diagnosis and more accurate monitoring of bone loss and BMD changes. By understanding the interactions among these omics data, personalized prevention and treatment strategies can be developed for osteoporosis.

7.2.3 Introduction of new tools to improve doctor-patient communication and treatment uptake for osteoporosis

To address the challenges of treating osteoporosis effectively, it is crucial to improve how healthcare providers communicate the seriousness of the condition and the need for treatment to patients. Conventional statistical metrics used in research can be complex and difficult for patients to understand, making it challenging for them to fully grasp the severity of osteoporosis and the importance of adhering to treatment.

The “Heat Age” concept has been widely used in cardiovascular risk communication, while the “Lung Age” metric has successfully encouraged smoking cessation [323-325]. Inspired by these tools, the concept of “Skeletal Age” was recently developed to improve communication of fracture-related mortality risk in osteoporosis patients [326]. By presenting skeletal gaining in a more relatable and understand way, this tool helps patients better appreciate their fracture risk and increases their willingness to follow treatment recommendations.

Similar patient-centered tools could be developed to further enhance communication about bone loss and its associated risks. By making these risks more understandable and personally relevant, such tools may encourage individuals to take treatment or prevention more seriously, support earlier intervention, and ultimately reduce both mortality and the personal and societal costs of osteoporosis.

7.2.4 Utilization of diverse populations to enhance the accuracy of risk prediction models

Utilizing large datasets and fostering collaboration can help uncover patterns in bone loss and BMD changes across diverse populations more effectively. In GWAS studies, some SNPs significantly associated with BMD in Taiwanese or Korean populations were not found in the Vietnamese population (as discussed in Chapter 6). Moreover, novel SNPs identified in one study need validation in different cohorts to reduce the likelihood of false-positive associations, given that GWAS tests a large number of SNPs. Replicating results strengthens the evidence for genetic associations and ensures that findings are not due to chance or sample biases, underscoring the need for collaboration among studies from different countries.

Furthermore, most osteoporosis research has primarily focused on postmenopausal Caucasian women, with less attention given to Caucasian men and non-Caucasian populations. Recognizing this limitation highlights the importance of including these diverse groups to investigate how varied dietary habits, exercise patterns, and cultural practices influence bone health.

7.2.5 Optimization of personalized lifestyle interventions tailored to genetic profiles

Future research should continue to explore how specific lifestyle factors, including dietary choices, physical activities, and other modifiable factors, can be optimized to mitigate bone loss and maintain or increase BMD. While genetic predisposition accounts for 50-80% of the variability in osteoporosis susceptibility, these inherited factors are unchangeable. In contrast, lifestyle choices can be modified for intervention.

These research directions are important for understanding, predicting, and preventing bone loss and changes in BMD, which are vital to managing osteoporosis effectively and reducing fracture risk and mortality. This approach aligns with the principle that prevention is preferable to treatment, highlighting proactive measures in maintaining bone health.

7.3 Conclusion

The increasing prevalence of osteoporosis with population aging cannot be ignored, especially given that it is a progressive condition rather than a discrete or dichotomous disease. Furthermore, osteoporosis is multifactorial, with the genetic basis of bone mineral density being polygenic. As a result, assessing osteoporosis risk requires simultaneous consideration of multiple factors, including age, genetics, and environmental influences.

The development of Polygenic Risk Scores, Genome-Wide Association Studies, and other omics data, along with recognition of diversity's importance in osteoporosis research, contribute to a more individualized understanding of osteoporosis risk. This approach aims to enhance early osteoporosis detection and improve prevention and treatment strategies.

Current discussions on improving osteoporosis risk assessment focus on three key areas: developing new predictive tools, researching lifestyle factors, and fostering inter-study collaboration to enhance early detection and improve preventive strategies, reducing the burden of osteoporosis on individuals and healthcare systems.

7.4 Limitations and strategies to overcome them

While this PhD study provided valuable insights into genetic factors and lifestyle influences on osteoporosis, certain limitations must be acknowledged. First, the study focused primarily on Caucasian populations in some cohorts, which may limit the generalizability of the findings to other ethnic groups. Future studies should aim to include more diverse populations to validate genetic associations across different backgrounds.

The reliance on self-reported data for lifestyle factors such as smoking, alcohol consumption, calcium, and dietary protein intake introduces potential recall and reporting biases, potentially affecting data accuracy. Additionally, categorizing these variables as categorical rather than continuous may have underestimated their effects, especially for heavy drinking or high cigarette use. Future research could incorporate objective measures, such as biochemical markers or wearable technology, to improve data precision. While adjustments were made for BMI, total energy intake was not considered, which might have influenced observed associations. Other factors like environmental conditions and vitamin D intake were not evaluated, leaving gaps in understanding the full range of influences on bone loss.

The limited availability of multiple BMD measurements (at least 3) in the VOS dataset restricted the ability to comprehensively study bone loss dynamics and conduct detailed longitudinal analyses. While the study explored genetic associations with BMD, its reliance on existing datasets meant that only a subset of potential genetic variants was analyzed. Expanding future research to include whole-genome sequencing could uncover additional variants contributing to BMD and osteoporosis risk, providing a more complete genetic profile.

While the Cox proportional hazards model is suitable for estimating mortality risk based on longitudinal BMD data, a limitation of this study is that covariates such as BMI, smoking and

drinking status, medication use, and comorbidities were only measured at baseline. Changes in these variables during follow-up were not accounted for, which may introduce residual confounding. Future studies incorporating time-varying covariates could provide a more accurate estimate of the relationship between bone loss and mortality risk. Additionally, the study's analysis of bone loss used mixed-effects models, which, while robust, may not capture all complexities of bone density changes over time. Incorporating machine learning approaches could improve the predictive accuracy of bone loss trajectories, especially when integrating multi-omics data.

Appendix A - Association between Sp1-binding-site polymorphism in the collagen type 1 alpha 1 (COLIA1) gene and bone phenotypes: The Dubbo Osteoporosis Epidemiology Study

The findings on the association between the COLIA1 gene and bone phenotypes—BMD, bone loss, and fracture—discussed in Chapter 5 have been published in the *Journal of Bone and Mineral Metabolism* during my doctoral study.

Huynh, N., De Dios, K., Tran, T. S., Center, J. R., & Nguyen, T. V. (In press). Association between Sp1 Binding Site Polymorphism in the collagen type 1 alpha 1 (COLIA1) gene and bone phenotypes: The Dubbo Osteoporosis Epidemiology Study. *Journal of Bone and Mineral Metabolism*. https://doi.org/10.1007/s00774-024-01558-8

Appendix B - Supplement of Chapter 3

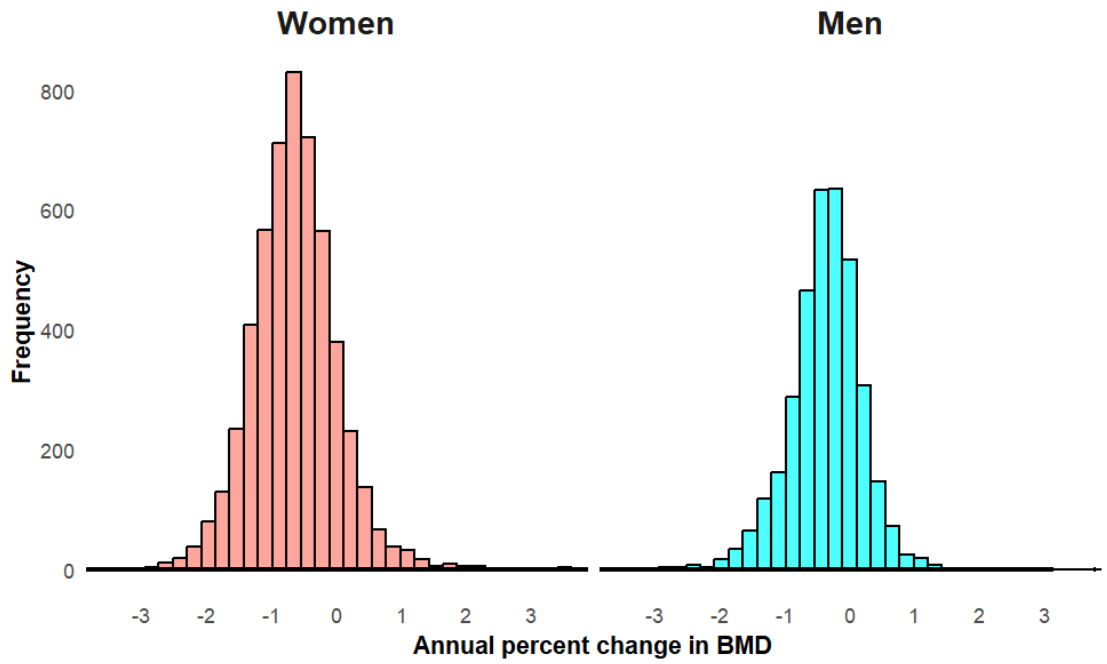

Figure S3.1 Distribution of percentage change in bone mineral density (BMD) at the femoral neck in women and men

Table S3.1 Association between lifestyle factors to variance of bone change in older women and men

		Women (N=5,288)		Men (N=3,557)	
Parameter	Unit	P-value in univariate linear regression analysis	P-value in multiple linear regression analysis*	P-value in univariate linear regression analysis	P-value in multiple linear regression analysis*
Age	Year	<0.001	<0.001	<0.001	<0.001
Smoking status	Non-smoker vs Smoker	<0.001	<0.001	0.015	0.001
Drinking status	No vs moderate**	<0.001	<0.001	0.146	0.094
Calcium intake[1]	Log scale	<0.001	<0.001	0.015	0.006
	Q1 vs Q4	<0.001	<0.001	0.039	0.013
Protein intake[2]	Log scale	0.12	0.96	0.296	0.615
	Q1 vs Q4	0.37	0.72	0.121	0.289
Physical activity[3]	Log scale	0.09	0.11	<0.001	<0.001
	Q1 vs Q4	0.39	0.38	<0.001	<0.001

*Multiple linear regression analysis was adjusted for age, BMI, any use of osteoporosis medication (fluoride, calcitonin, bisphosphates such as alendronate and etidronate, thyroid hormone, or estrogen), and concomitant diseases (cardiovascular diseases, cancer, or osteoporosis) in women, and additional adjusted for race in men.

** Moderate in drinking status was considered ≥1-<7 drinks/week in women, and ≥7-<14 drinks/week in men.

[1] For calcium intake, values are presented as quartiles (Q) with ranges defined as follows: In 4243 women, Q1: ≤698, Q2: 699-1198, Q3:1199-1719, Q4: ≥1720, and in 3557 men, Q1: ≤687, Q2: 688-1010, Q3: 1011-1572, Q4: ≥1573)

[2] For protein intake, values are presented as quartiles (Q) with ranges defined as follows: In 2915 women, Q1: ≤41, Q2: 42-51, Q3: 52-60, Q4: ≥61, and in 2897 men, Q1: ≤44, Q2: 45-56, Q3: 57-68, Q4: ≥69)

[3] For physical activity, values are presented as quartiles (Q) with ranges defined as follows: In 3605 women, Q1: ≤954, Q2: 955-1538, Q3:1539-2497, Q4: ≥2498, and in 3114 men, Q1: ≤1944, Q2: 1945-2218, Q3:2219-2520, Q4: ≥2521)

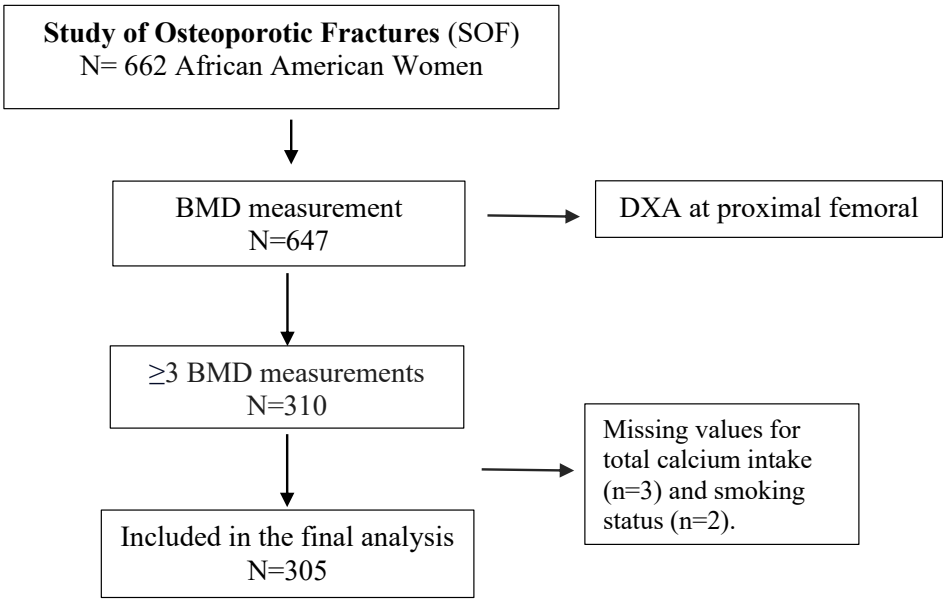

Figure S3.2 Flow chart of SOF African American participants according to the analytical samples. Abbreviations: BMD, bone mineral density. *Missing value for age (n=33), weight (n=61), height (n=38), and BMI (n=26) were replaced with the mean value of each respective variable.

Table S3.2 Characteristics of study participants in SOF by ethnicity

Baseline characteristics	**White women N = 5,288**	**Black women N = 305**	**P-value**
Age (yrs)	72 (4)	75 (3)	<0.001
Weight (kg)	66.8 (11.4)	75.6 (10.8)	<0.001
Height (cm)	160 (5.6)	159 (4.7)	0.001
BMI (kg/m^2)	26.2 (4.2)	30.0 (4.2)	<0.001
Femoral neck BMD (g/cm^2)	0.66 (0.11)	0.75 (0.14)	<0.001
Smoking status			0.901
Smoker	413 (7.8 %)	22 (7.2%)	
Ex-smoker	1601(30.3 %)	95 (31.1%)	
Non-smoker	3274 (61.9%)	188 (61.6%)	
Drinking status			
0 drink/week	2170 (41.1 %)	212 (69.5%)	
<1 drink/week	1400 (26.5 %)	51 (16.7%)	
≥1-<7 drinks/week	1121 (21.3%)	31 (10.2%)	
≥7-<14 drinks/week	414 (7.82%)	8 (2.6%)	
≥14-<21 drinks/week	140 (2.65%)	2 (0.7%)	
≥21 drinks/week	43 (0.81%)	1 (0.3%)	
Total calcium intake (mg/day)	1243 (630)[a]	920 (556)	<0.001
Dietary protein intake (g/day)	50.9 (13.7)[b]	51.4 (16.7)[b]	0.627
Physical activity (kcal/day)	1827 (1070)[c]	1355 (916)[c]	<0.001

Values are presented as mean (SD) unless otherwise specified. [a] Only 4246 White women with total calcium intake were included. [b] Only 2915 White women and 241 Black women with dietary protein intake were included. [c] Only 3605 White women and 87 Black women with physical activity levels were included in the analysis.

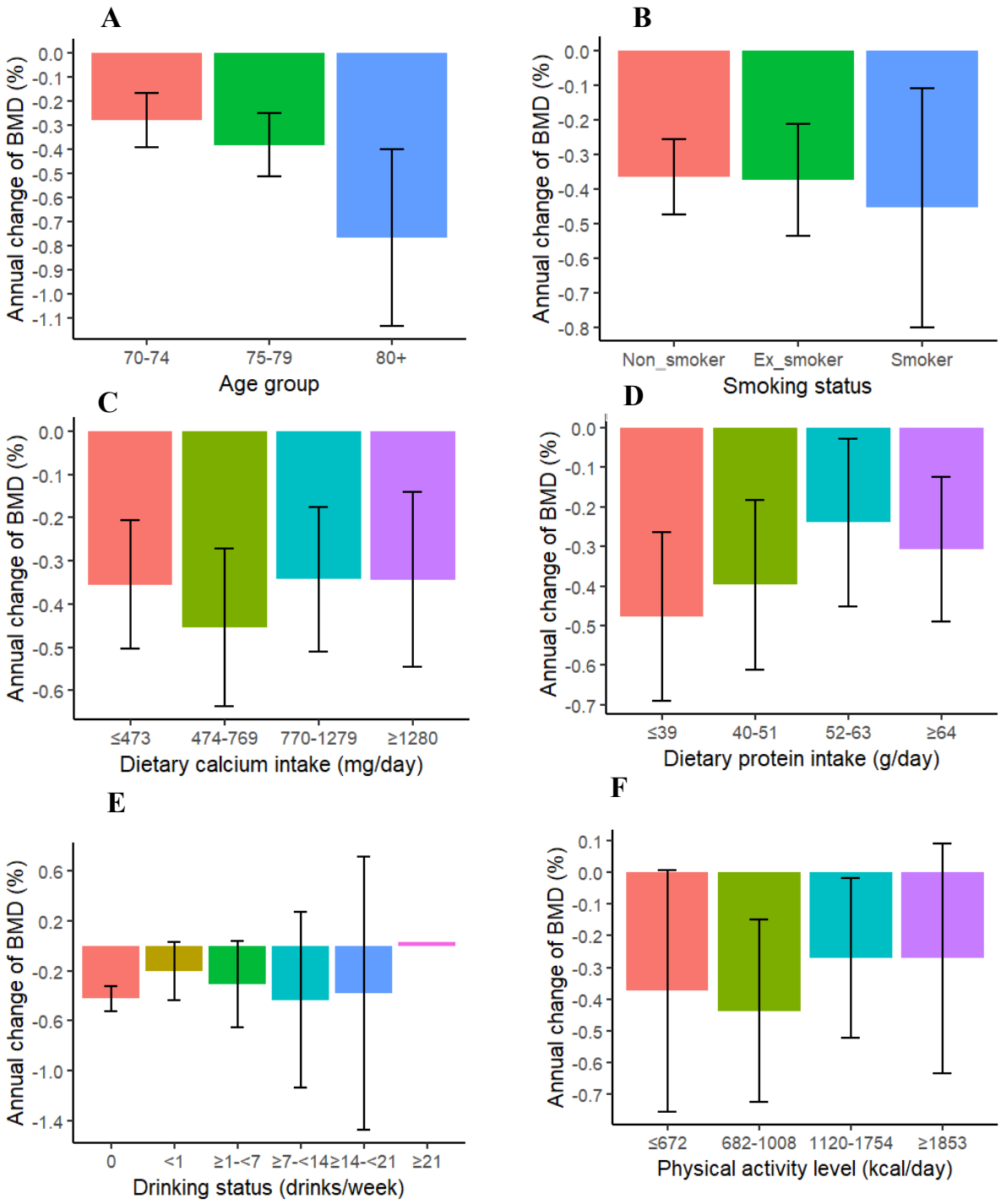

Figure S3.3 Annual percentage change in bone mineral density (BMD) at the femoral neck, stratified by age groups (A), smoking status (B), quartile of total calcium intake (C) in 305 Black women, quartile of dietary protein intake (D) in 250 Black women, drinking status (E) in 305 Black women, and quartile of physical activity level (F) in 87 Black women.

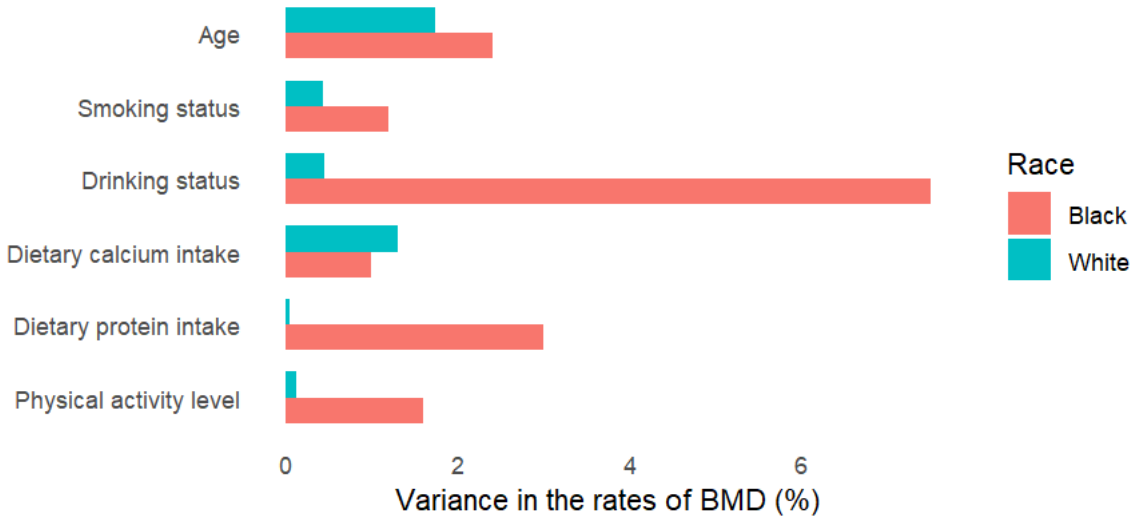

Figure S3.4 The percentage of variance in the rate of bone mineral density (BMD) in femoral neck contributed by age, smoking status, drinking status, total calcium intake, dietary protein intake, and physical activity level in 1940 White women and 66 Black women. Age was in years. Non-smoker was used as a reference for smoking status, and 0 drink/week was used as a reference for drinking status. Total calcium intake, dietary protein intake and physical activity were measured in a log scale.

Table S3.3 Contributions of lifestyle factors to variance of bone change in Black women

Predictive factor	Unit	Women (n=66)		
		Regression coefficient (standard error)	**P-value**	**Percent of contribution to variance in bone loss**
Age	1 year	-0.04 (0.031)	0.23	**2.4%**
Smoking	current vs. never	-0.37 (0.43)	0.39	**1.2%**
Alcohol	Drinking* vs. never	0.73 (0.45)	0.11	**7.5%**
Total calcium intake**	ln(mg/day)	0.04 (0.17)	0.79	**1.0%**
Dietary protein intake**	ln(g/day)	0.38 (0.28)	0.18	**3.0%**
Physical activity**	ln(kcal/day)	0.14 (0.17)	0.41	**1.6%**

Note: *Drinking status comparing ≥1-<7 vs never; ** in natural logarithmic scale.

Table S3.5 Association between lifestyle factors to variance of bone change in Black women

		Women (N=305)	
Parameter	Unit	P-value in univariate linear regression analysis	P-value in multiple linear regression analysis*
Age	Year	0.001	0.002
Smoking status	Non-smoker vs Smoker	0.607	0.328
Drinking status	No vs <1drink/week	0.065	0.007
Calcium intake[1]	Log scale	0.733	0.978
	Q1 vs Q4	0.962	0.642
Protein intake[2]	Log scale	0.358	0.675
	Q1 vs Q4	0.154	0.426
Physical activity[3]	Log scale	0.465	0.306
	Q1 vs Q4	0.642	0.395

*Multiple linear regression analysis was adjusted for age, BMI, any use of osteoporosis medication (fluoride, calcitonin, bisphosphates (alendronate, etidronate), thyroid hormone, or estrogen), and concomitant diseases (cardiovascular diseases, all types of cancer, or osteoporosis).

[1] For calcium intake, values are presented as quartiles (Q) with ranges defined as follows: In 305 Black women, Q1: ≤473, Q2: 474-769, Q3:770-1279, Q4: ≥1280

[2] For protein intake, values are presented as quartiles (Q) with ranges defined as follows: In 241 Black women, Q1: ≤39, Q2: 40-51, Q3: 52-63, Q4: ≥64.

[3] For physical activity, values are presented as quartiles (Q) with ranges defined as follows: In 87 Black women, Q1: ≤672, Q2:682-1008, Q3:1120-1498, Q4: ≥1754

Appendix C - Supplement of Chapter 4

Table S4.1 Characteristics of men stratified by ethnicity groups

Characteristics	Caucasian (N=3231)	African American (N=2746)	Asian (N=115)	Other (N=98)	*P*-value
Age (yrs)	72 (5)	70 (4)	72 (5)	71 (5)	<0.001
Height (cm)	175 (6.5)	175 (7.5)	167 (5.9)	173 (7)	<0.001
Weight (kg)	84 (12.5)	87 (15)	71 (9.7)	83 (15)	<0.001
BMI (kg/m^2)	27.4 (3.6)	28.4 (4.1)	25.3 (3.3)	28 (4.2)	<0.001
Smoking status: Current Past Never	86 (2.7%) 1870 (57.8%) 1276 (39.5%)	12 (10.6%) 61 (54.0%) 40 (35.4%)	2 (1.7%) 63 (54.8%) 50 (43.5%)	5 (5.1%) 50 (51.0%) 43 (43.9%)	-
Alcohol status: Drinker	2258 (69.9%)	64 (56.6%)	57 (49.6%)	69 (70.4%)	<0.001
FNBMD (g/cm^2)	0.787 (0.126)	0.897 (0.151)	0.767 (0.119)	0.803 (0.118)	<0.001
Bone change	-0.444 (0.494)	-0.415 (0.499)	-0.320 (0.443)	-0.385 (0.455)	0.039
Rate: Unchanged Increased Decreased Rapid decrease	2841 (88%) 13 (0.4%) 357 (11.0%) 20 (0.6%)	99 (87.7%) 1 (0.8%) 13 (11.5%) 0 (0.0%)	107 (93.1%) 0 (0.0%) 8 (6.9%) 0 (0.0%)	89 (90.8) 0 (0.0%) 9 (9.2%) 0 (0.0%)	_
Medication: User	233 (7.7%)	5 (4.5%)	5 (4.5%)	3 (3.2%)	0.141
Comorbidities: At least one	1890 (58.5%)	80 (70.8%)	66 (57.4%)	54 (55.1%)	0.058
Death status: Deceased	2551 (78.9%)	85 (75.2%)	78 (67.9%)	67 (68.4%)	0.002

Values are presented as mean (SD) unless otherwise specified. FNBMD: femoral neck bone mineral density.

Appendix D - Supplement of Chapter 6

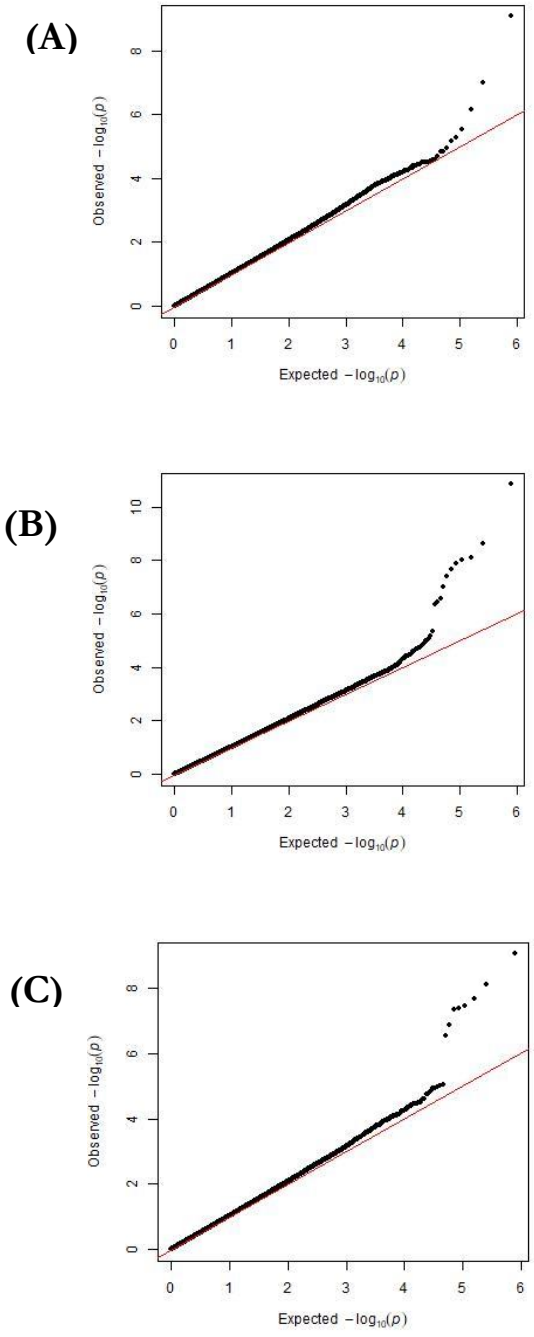

Figure S6.1 Quantile-quantile plots for genome-wide association analysis of bone mineral density at the lumbar spine (A), femur neck (B), and total hip (C). The plots show expected versus observed p-values from LMM analysis, both log-transformed, for 386,455 SNPs.

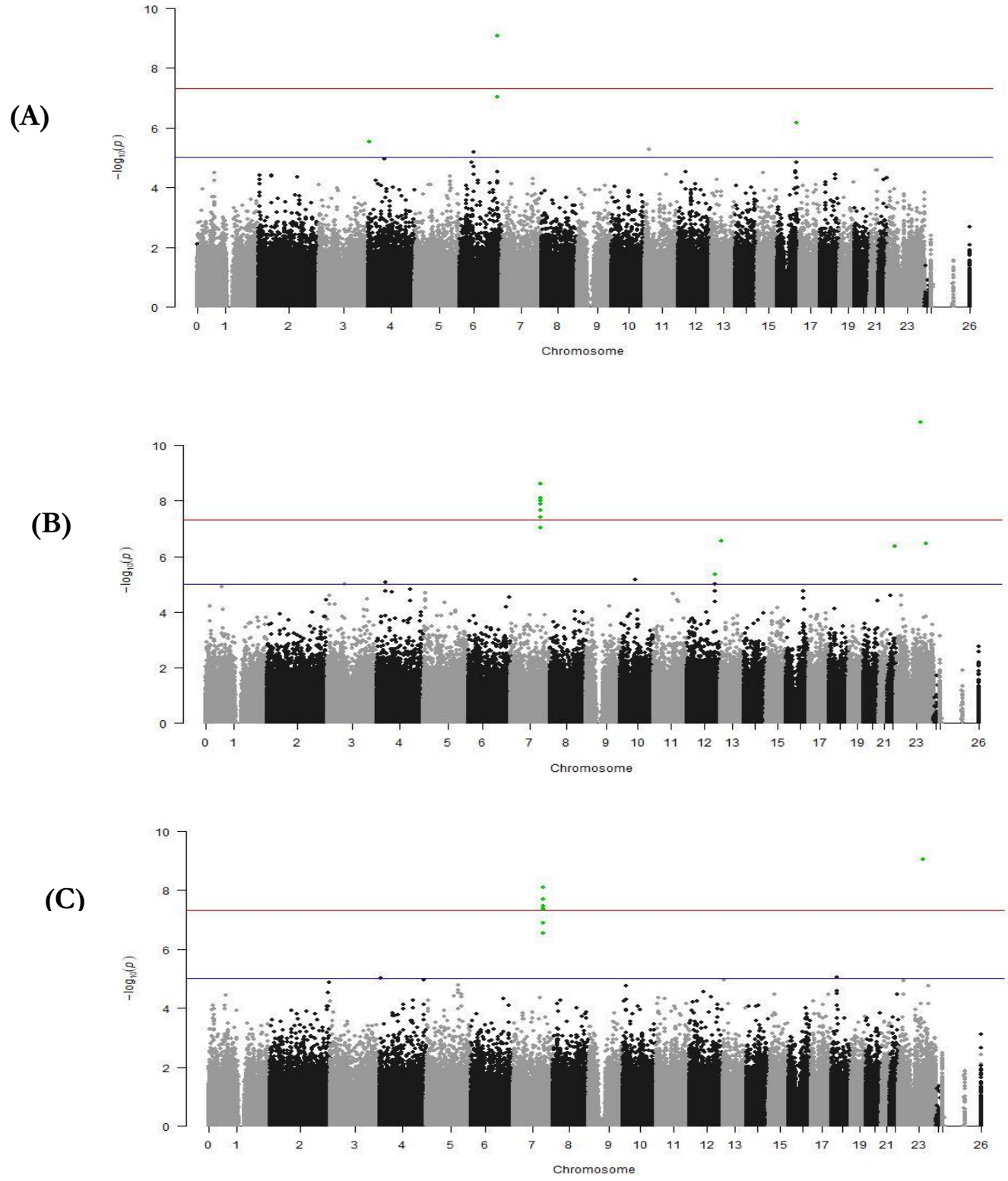

Figure S6.2 Manhattan plot. (A) Manhattan plot of bone mineral density (BMD) at the lumbar spine. (B) Manhattan plot of BMD at femur neck. (C) Manhattan plot of BMD at total hip.

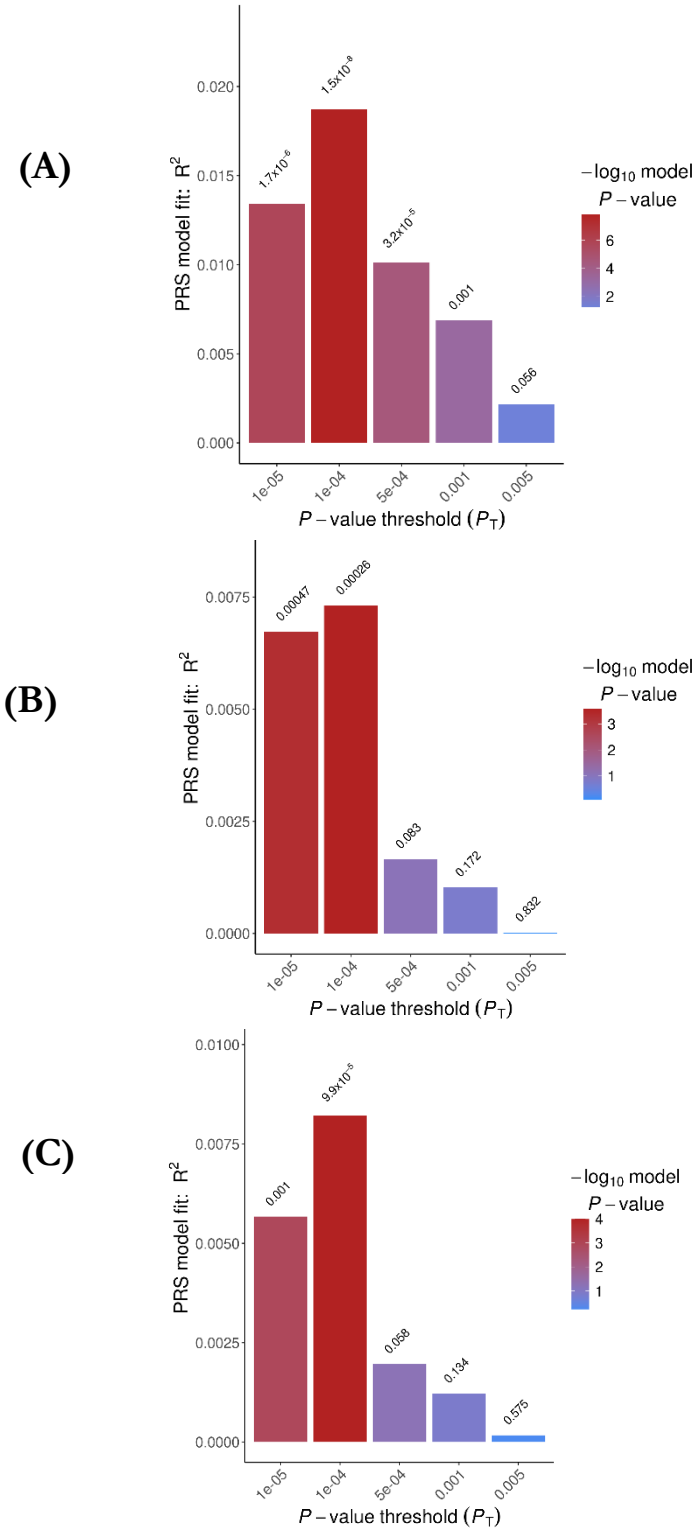

Figure S6.3 Bar plot from PRS-CS showing results at broad P-value thresholds for bone mineral density (BMD) at the lumbar spine (A), at femur neck (B), and at total hip (C).

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