Epidemiological investigations of biomarker and dietary relationships with osteoporosis and fracture risk

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Abstract

Diet may aid osteoporosis and fracture prevention as it is modifiable, but limited evidence for a role of vitamin K_1, vitamin C and iron exists, despite suggestions of potential underlying mechanisms. Positive associations between these nutrients and bone health have been reported in population studies; however, evidence is scarce in men, in British populations, for nutrient status and for fracture risk. Combining measures of dietary intake with biomarkers may limit errors associated with establishing population intakes, but no such studies exist in bone health. Therefore, this thesis aimed to investigate micronutrient intakes and blood measures and i) their cross-sectional associations with heel ultrasound and ii) their prospective associations with fracture risk in a sub-set of EPIC-Norfolk participants. An additional aim was to explore means of limiting the impact of measurement errors on the association between vitamin C and bone health. The main results showed significant associations between higher intakes of vitamin K_1 and C and 0.6-5.5% higher heel ultrasound in both sexes, and additionally with total and plant-based iron intakes in women (0.4-5.8%). Moreover, upper *versus* lower quintiles of plasma vitamin C concentrations in men showed significant associations with reduced fracture risk at the hip (HR:0.35, 95%CI:0.16-0.80) and spine (HR:0.26, 95%CI:0.10-0.69). In women, upper *versus* lower quintiles of vitamin K_1 intake (HR:0.47, 95%CI:0.24-0.91), total iron intake (HR:0.41, 95%CI:0.21-0.79), animal-based iron intake (HR:0.44, 95%CI:0.24-0.82) and serum ferritin concentrations (HR:0.30, 95%CI:0.14-0.64) were significantly inversely associated with spine fracture risk. In contrast, upper *versus* lower quintiles of animal iron intake in men was significantly associated with higher hip fracture risk (HR:2.29, 95%CI:1.11-4.73). . In further investigations, combining vitamin C intake and plasma status strengthened the associations with bone health in men, but not in women. In conclusion, this thesis provides novel insights into the role of diet in osteoporosis and fracture prevention.

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List of abbreviations

7dDD	7-day diet diary
BMD	Bone mineral density
BMI	Body mass index
BSALP	Bone-specific alkaline phosphatase
BUA	Broadband ultrasound attenuation
CRP	C-reactive protein
CTx/NTx	Collagen type 1 cross-linked C-telopeptide / N-telopeptide
DINER	Data into Nutrients for Epidemiological Research
DPA	Dual-photon absorptiometry
DPD	Deoxypyridinoline
DXA	Dual-energy x-ray absorptiometry
ENCORE	East Norfolk health authority database
EPIC	European Prospective Investigation into Cancer and Nutrition
F&V	Fruit and vegetable intake
FFQ	Food frequency questionnaire
FN	Femoral neck
GT	Greater trochanter
HRT	Hormone replacement therapy
IHD	Ischemic heart disease
IL-6	Interleukin-6
iT	Intertrochanter
LS	Lumbar spine
MDR	Mid-distal radius
NDNS	National Diet and Nutrition Survey
NHS	National Health Service
OC	Osteocalcin
OPG	Osteoprotegerin
PICP/PINP	Procollagen type I C-terminal / N-terminal propeptide
PPAR-γ	Peroxisome proliferator-activated receptor gamma
PYD	Pyridinoline
R	Radius
RCT	Randomised controlled trial
RS	Radial shaft
PYR	Pyridinoline

RANK(L)	Receptor activator of nuclear factor kappa-B (ligand)
RNI/LRNI	Reference Nutrient Intake / Lower Reference Nutrient Intake
T	Trochanter
TH	Total hip
TNF-α	Tumor necrosis factor alpha
ucOC	Undercarboxylated osteocalcin
UDR	Ultradistal radius
ViMiS	Vitamin and mineral supplement database
VOS/SOS	Velocity of sound / Speed of sound
WB	Whole body
WHO	World Health Organization
WT	Ward's triangle

List of publications

Finck H, Hart AR, Lentjes MAH, Jennings A, Luben RN, Khaw K-T and Welch AA (2015) Cross-sectional and prospective associations between dietary and plasma vitamin C, heel bone ultrasound, and fracture risk in men and women in the European Prospective Investigations into Cancer and Nutrition in Norfolk cohort (in press). *The American Journal of Clinical Nutrition* **102**:1-9.

Finck H, Hart A, Lentjes M, Jennings A, Luben R, Khaw K-T and Welch A (2015) Prospective associations between dietary iron intake and serum ferritin concentrations with fracture risk in EPIC-Norfolk men and women (abstract). *Proceedings of the Nutrition Society* **74(OCE2)**, E184.

Finck H, Hart AR, Jennings A and Welch AA (2014) Is there a role for vitamin C in preventing osteoporosis and fractures? A review of the potential underlying mechanisms and current epidemiological evidence. *Nutrition Research Reviews* **27(2)**:268-283.

Finck H, Hart AR, Lentjes MA, Jennings A, Luben RN, Khaw K-T and Welch AA (2014) Associations between vitamin C and quantitative heel ultrasound and spine fracture risk (abstract). *Osteoporosis International* **25(Supp 2)**, P129.

Finck H, Cassidy A, Lentjes M, Jennings A, Luben R, Khaw K-T and Welch A (2013) Dietary vitamin C is positively associated with heel bone density but not with fracture risk in men and women in the EPIC-Norfolk study (abstract). *Proceedings of the Nutrition Society* **72(OCE4)**, E254.

List of presentations

Finck H, Hart A, Lentjes M, Jennings A, Luben R, Khaw K-T and Welch A. *Prospective associations between dietary iron intake and serum ferritin concentrations with fracture risk in EPIC-Norfolk men and women.* (Oral presentation, Nutrition Society Winter Meeting, December 2014, London, UK)

Finck H, Hart A, Lentjes M, Jennings A, Luben R, Khaw K-T and Welch A. *Is there a role for iron in preventing fractures?* (Oral presentation, Norwich Research Park Diet and Health Tea Club Meeting, September 2014, University of East Anglia, UK)

Finck H, Hart A, Lentjes M, Jennings A, Luben R, Khaw K-T and Welch A. *Associations between vitamin C and quantitative heel ultrasound and spine fracture risk.* (Poster presentation, World Congress on Osteoporosis, Osteoarthritis and Musculoskeletal Diseases (WCO-IOF-ESCEO), April 2014, Seville, Spain)

Finck H, Cassidy A, Lentjes M, Jennings A, Luben R, Khaw K-T and Welch A. *Dietary vitamin C intake, heel bone density and fracture risk.* (Oral presentation, Nutrition Society Summer Meeting, July 2013, University of Newcastle, UK)

Finck H, Cassidy A, Jennings A, Khaw K-T and Welch A. *Are dietary vitamin C and K_1 important for bone health?* (Oral presentation, Postgraduate Research Student Conference of the Faculty of Medicine and Health Sciences, March 2013, University of East Anglia, UK)

Finck H, Cassidy A, Jennings A and Welch A. *Osteoporosis: Another reason to eat more fruits and vegetables.* (Poster presentation, Postgraduate Research Showcase to the community in a public library space, June 2012, Norwich, UK)

Finck H, Cassidy A, Jennings A, Macgregor A and Welch A. *Vitamin C and bone health.* (Poster presentation, Postgraduate Research Student Conference of the Faculty of Medicine and Health Sciences, March 2012, University of East Anglia, UK)

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CHAPTER 1

INTRODUCTION

1.1 Introduction

The pathology of osteoporosis and osteoporotic fractures is still not fully understood [1]. Consequently, specific recommendations regarding the reduction in risk are limited [2]. Diet is an important lifestyle factor which may modify risk and thus provide a useful strategy in the prevention of osteoporosis and fractures. To date, associations between specific dietary compounds and bone health are limited [3-6] and further investigations are warranted. Micronutrients including iron (from animal- and plant-based food sources) and vitamin C might exert beneficial effects on bone through their role in the formation and maintenance of bone collagen [7-10]; and vitamin K_1 through the γ-carboxylation of osteocalcin in bone [11]. Thus, higher dietary intakes of iron and the vitamins C and K_1 could provide a strategy for the prevention of osteoporosis and associated fractures.

1.2 Osteoporosis

1.2.1 Bone biology

The skeleton is the vital supporting framework of the human body to which other organs and muscles connect. It is a rigid supporting structure as bones are almost as strong as cast iron despite being approximately ten times more flexible and three times lighter[12]. The skeletal system is also crucial for the protection of vital organs including the brain through the skull, and the heart and lungs through the rib cage. It works in tight conjunction with the muscular system to allow for movement and the circulatory system to enable nutrient exchange and the synthesis of blood cells including erythrocytes, leukocytes and thrombocytes. Bone is comprised of the inorganic phase (65%), which is predominantly associated with its strength and stiffness, the organic phase (25%), which contributes to its strength and is pre-dominantly composed of type I collagen, and water (10%) [12, 13].

1.2.2 Bone remodeling

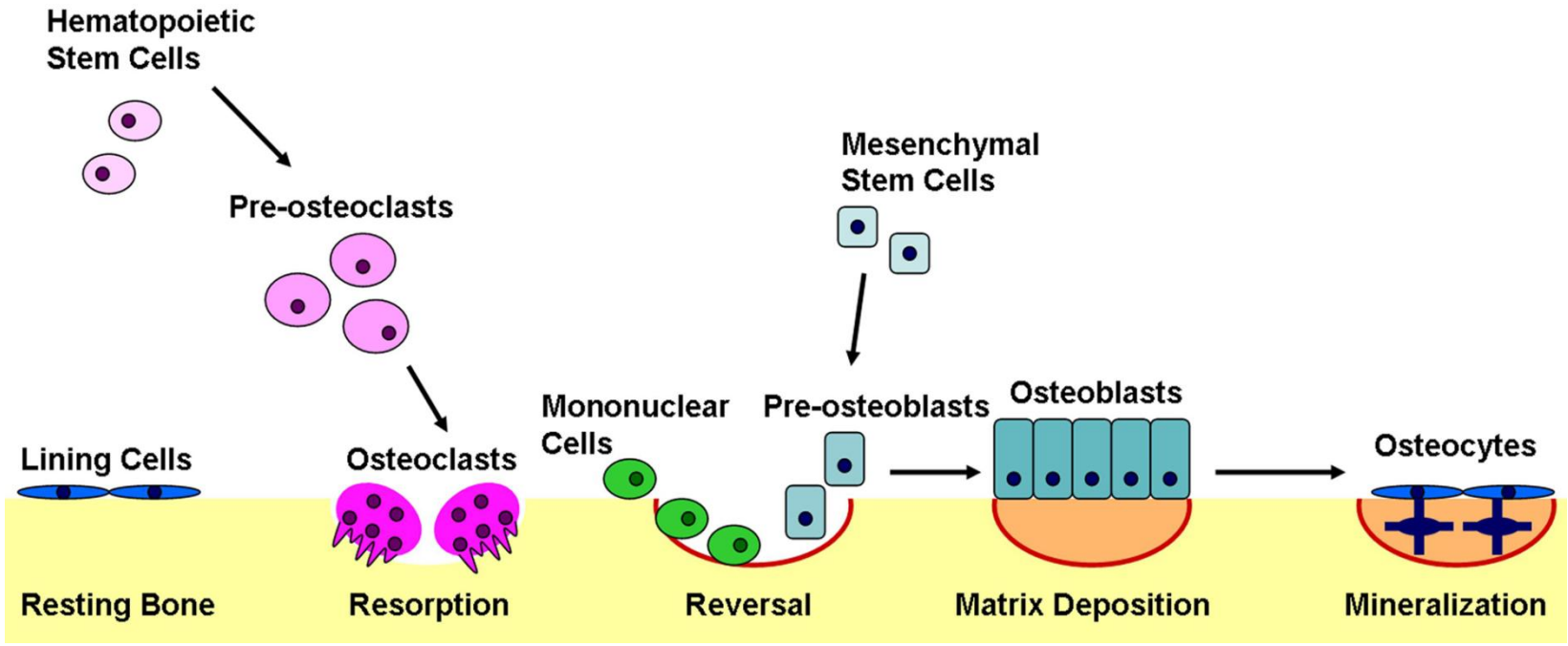
The skeleton is a very metabolically active organ with a continuous turnover of bone material. This remodeling process is essential to overall bone health as it allows for repairing damaged bone, responding to variation in metabolic demands (such as maintaining blood calcium concentrations) and adapting to changes in mechanical load and strain [14, 15]. Bone remodeling is characterised by the degradation of bone by osteoclasts (bone resorption) and the synthesis of new bone tissue by osteoblasts (bone formation) **(Figure 1.1)**. Initially, osteoclasts create cavities on the surface of bone. The maturation of osteoclast precursors and activation of mature cells is partly governed by the signalling pathway of receptor activator of nuclear factor kappa-B and its ligand (RANK/RANKL). RANKL, produced by the bone forming osteoblasts, stimulates

osteoclastogenesis by binding to its receptor RANK located on the osteoclast precursors and mature cells [16]. Osteoblasts are attracted to the newly formed cavities and then start depositing bone extracellular matrix which involves synthesising and secreting type I collagen, as well as mineralising newly formed bone tissue [17]. Osteoblast differentiation may partly be mediated by the expression of peroxisome proliferator-activated receptor gamma (PPAR-γ), an essential transcription factor in adipogenesis [18]. The process of bone remodeling at any one site may take between three and six months, and most of this time represents the synthesis of bone [19, 20].

1.2.3 Changes in bone mass over a lifetime

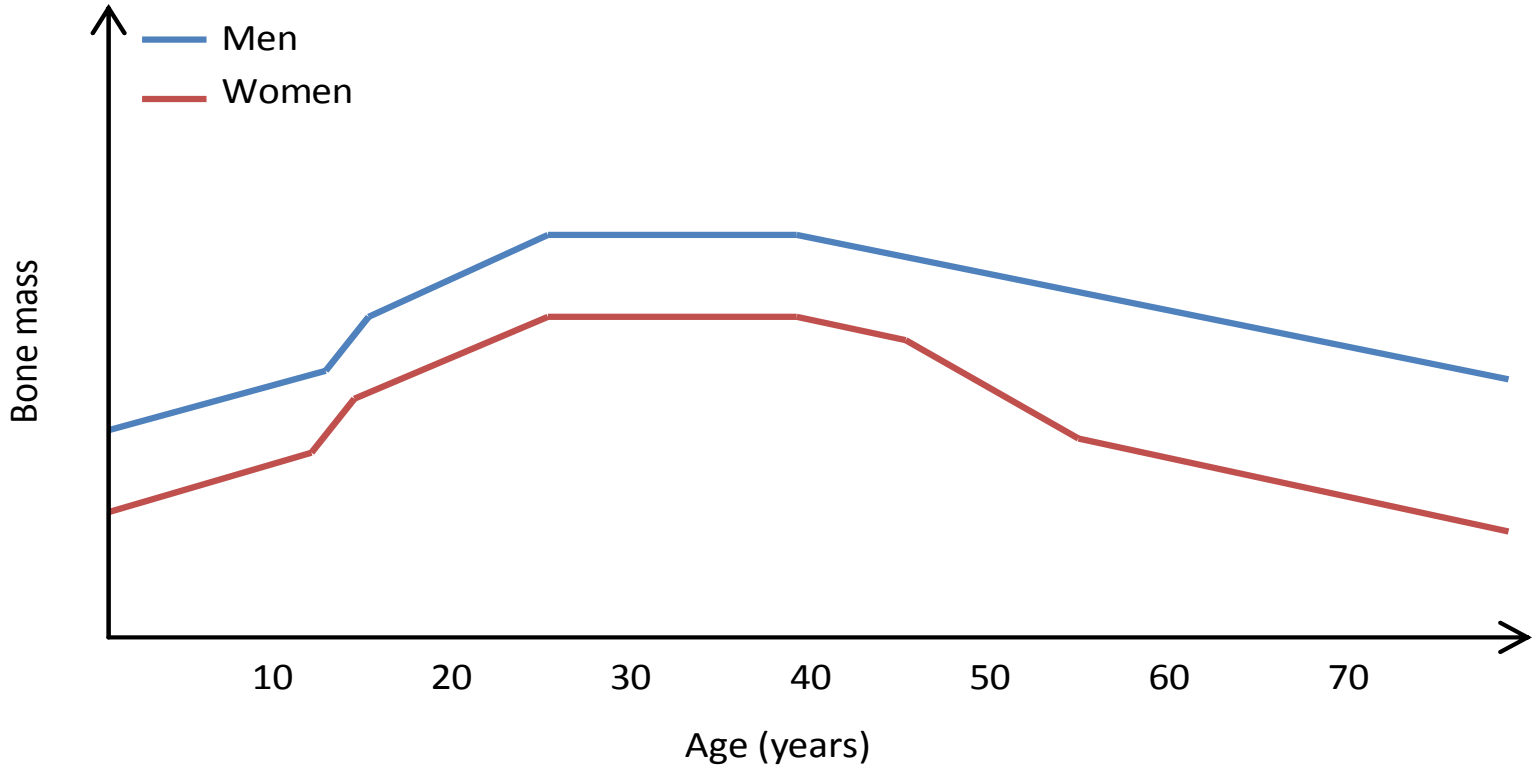
Bone remodeling is a continuous lifelong process of bone repair and growth **(Figure 1.2)**, and is highly dependent on physiological demands. In early and pubertal life, bone remodeling is at an imbalance in favour of bone formation. This is in response to growth spurts associated with childhood and adolescence and is crucial for the elongation of bones. Between the ages of 25-40 years, there is equilibrium between bone formation and bone resorption. Age-related bone loss starts at around 40 years of age, possibly as a result of an increased bone resorption rate, and is an ongoing process until death. Decreased bone mass is associated with significantly greater fracture risk of varying degree depending on the bone site [21]. Consequently, the risk of developing osteoporosis and associated fractures increases with age and is greatest in the elderly population [22]. The average rate of bone loss is approximately 0.7-0.8% per year [23-25], except during the female menopausal transition where the low oestrogen levels result in an accelerated loss of bone mass of up to 2% per year [26]. Menopausal bone loss, as well as the fact that women have lower bone mass than men throughout life due to their smaller body size, infers great sex differences in bone health, with women having a higher risk of developing osteoporosis and fractures than men [27].

Figure 1.1: Bone remodeling.



From Kapinas & Delany (2011) [28].

Figure 1.2: Simulation of the changes in bone mass over a lifetime by sex.

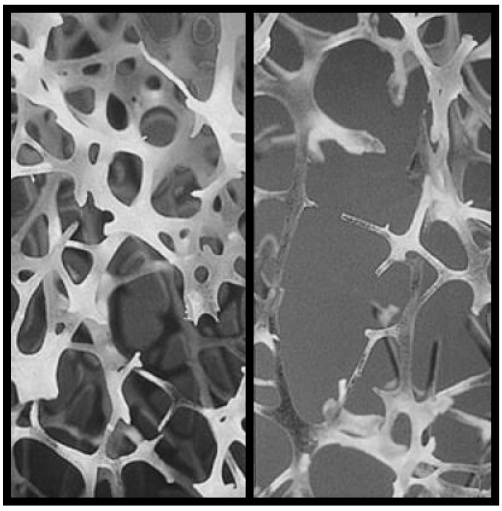


Adapted from Compston (1990) [29].

1.2.4 Osteoporosis and its underlying mechanisms

Age-related loss of bone mass is not reversible and has previously been associated with lower levels of bone mineral density (BMD) [30, 31]. These changes make bone thin, porous and brittle and may eventually lead to osteoporosis in old age. Osteoporosis is characterised by a reduction in the amount of bone tissue and structural changes in the bone matrix, such as a lesser degree of mineralisation **(Figure 1.3)**. These changes are not associated with any signs or symptoms, and consequently the disease often progresses for prolonged periods of time without the individual noticing. The age at which low bone density reaches the diagnostic threshold for osteoporosis differs between individuals, but most commonly occurs in later life.

Figure 1.3: A comparison of healthy bone (left) with osteoporotic bone (right).



Adapted from National Osteoporosis Society, UK.

The underlying mechanisms of osteoporosis are complex and not yet fully understood. It is thought that a number of risk factors including increasing age may influence the natural balance of bone remodeling towards greater bone resorption, possibly via interfering with calcium homeostasis, natural hormone concentrations and increasing oxidative stress. In previous experimental studies, free radicals were shown to be involved in osteoblastogenesis, apoptosis of osteoblasts and osteoclastogenesis [32-34]. For example, an *in vitro* study showed that the formation of osteoblasts was inhibited by free radicals, although the underlying mechanisms are not yet fully understood [35]. Furthermore, findings from an *in vivo* study in mice found that free radicals increase bone resorption through the activation of nuclear factor-κB [36], a transcription factor for genes involved in the survival, differentiation, inflammation and growth of cells [37]. Previous experimental studies have also shown that osteoclasts naturally produce reactive oxygen species to facilitate the destruction of calcified tissue during bone resorption, hence playing an important role in bone remodeling [38, 39]. The exposure to risk factors may shift the

natural equilibrium between oxidants and antioxidants leading to excessive bone loss and a consequent increased risk for osteoporosis and fractures [40].

Another suggested underlying mechanism for the development of osteoporosis is the modification of the OPG/RANKL pathway through its interaction with factors such hormones, cytokines, growth factors and vitamins [16]. Naturally, these modulators are involved in osteoclastogenesis and bone remodeling via tightly regulating the balance of RANKL/RANK and osteoprotegerin (OPG). However, modifications of modulators such as age-related changes in oestrogen levels in women may lead to increased expression of RANKL and reduced OPG secretion and consequent reduction in BMD [16].

In the past few decades, a link between systemic inflammation, which is commonly present at low levels in older age [41, 42], and a higher rate of bone turnover and decreased bone mass was suggested [43]. In any inflammatory state, circulating levels of pro-inflammatory cytokines including interleukin-6 (IL-6) and tumor necrosis factor alpha (TNF-α) are elevated [44], and this has previously been associated with increased bone resorption and greater bone loss [45-48]. For example, IL-6 has been shown to increase osteoclast activity by suppressing osteoclast apoptosis [49]. Moreover, circulating levels of C-reactive protein (CRP), a sensitive marker of systemic inflammation, were associated with low BMD [50]. In a cross-sectional study of 4693 pre- and postmenopausal women, CRP levels were higher in osteoporotic patients compared to normal subjects, and the odds ratio for osteoporosis in postmenopausal women was 1.54 (95%CI 1.10-2.53) for those in the top compared to the bottom quintile of CRP levels [51]. Similarly, in a prospective study of 2985 older men and women, inflammatory markers measured at baseline were significantly higher in participants who subsequently experienced a fracture after the 6-year follow-up, and the relative risk of a fracture was 2.65 (95%CI 1.44-4.89) in subjects with three or more compared to no elevated inflammatory markers [52].

Another study suggested underlying mechanism in osteoporosis relates to disruptions in Wnt signalling which is the genetic encoding of a number of proteins such as glycoproteins that mediate a diverse range of processes including embryogenesis and tumorigenesis [53]. Such disruptions have previously been linked to a reduction in bone mass [54]. Under normal circumstances, Wnt proteins activate specific pathways leading to an increase in bone mass through stimulation of preosteoblast replication, induction of osteoblastogenesis and inhibition of osteoblast apoptosis [55]. Recently, a number of Wnt antagonists have been identified, including secreted frizzled related proteins [56], dickkopfs [57] and Sclerostin [58], which have been shown to inhibit a number of Wnt pathways, thus interfering with bone formation.

1.2.5 The assessment of osteoporosis

In 1994, the World Health Organization (WHO) defined osteoporosis as a BMD (g/cm^2) at the hip or spine of 2.5 standard deviations or more below the sex-specific young adult mean [59]. Dual-energy x-ray absorptiometry (DXA) is the gold standard for estimating BMD and is thus the most commonly used technique. It determines the average amount of bone mineral of the scanned area in a two dimensional format by measuring the difference in tissue attenuation between two X-ray beams of differing energy levels [60]. The exposure to radiation is very low and is comparable to one sixth of a chest radiation or one thirteenth of a conventional mammogram [61, 62]. The measurement of BMD in older adults is important for the early detection of osteoporosis, which otherwise tends to remain unexposed until a fracture occurs.

Other comparatively inexpensive, portable and radiation-free techniques for quantifying bone mass include the use quantitative ultrasound, although measurements are less precise than those using DXA. Ultrasound yields the parameters broadband ultrasound attenuation (BUA; in dB/MHz) and velocity of sound (VOS; in m/s). BUA measures bone density as well as its structural organisation by passing a short burst of ultrasound through the bone, typically the heel [63]. For this, it uses the attenuation of several frequencies of acoustic waves above the audible frequency range (typically defined as 20 kHz) [62]. VOS is a measure of both bone density and bone stiffness and takes into account the distance between the two transducers used during the measurement and the transit time of the signal between them [63]. Higher values for both BUA and VOS indicate greater bone quality. Although VOS has not been widely studied, it has previously been shown to have a relatively low coefficient of variation (CV) of 0.3-0.5% [64, 65], indicating a relatively high precision of measurement. In contrast, the CV of BUA has previously been shown to range between 2.0 - 4.0 % [65, 66] which is higher than that of DXA of 0.8 – 2.2 % [61], thus making it less precise than the X-ray technique. Despite these differences in measurement precision, ultrasound has previously been shown to be capable of distinguishing bone densities of subjects with and without osteoporosis [67], and age-related bone loss has also been demonstrated [64]. Moreover, several studies have indicated that ultrasound measurements predict the risk of osteoporotic fractures as well as DXA measurements [68-71]. However, ultrasound is currently not used as a diagnostic tool for osteoporosis, and thus the detection of low bone density using ultrasound must be followed up by further measurements using DXA.

Skeletal status may also be assessed using various biochemical markers of bone formation and bone resorption found in serum, plasma and urine [72]. Bone formation markers may indicate different stages of bone formation and individual aspects of osteoblast function and include bone-specific alkaline phosphatase (BSALP), osteocalcin (OC) and procollagen type I propeptides (PINP, PICP). Markers of bone resorption may be degradation products of collagen present in bone, bone proteins or enzymes including pyridinium crosslinks of collagen (PYD, DPD) and collagen type I telopeptides (CTx, NTx). However, bone turnover markers may only

indicate short-term changes in bone homeostasis and are prone to high levels of within-subject variability [73]. They are currently not used for the diagnosis of osteoporosis.

1.2.6 The prevalence of osteoporosis

Osteoporosis is a major cause of illness and death in the elderly population. It is a very prevalent condition in older people, particularly older women, and has been estimated to affect 75 million people in Europe, Japan and the United States [74]. In the UK alone, almost three million people are estimated to have osteoporosis [75]. Given the ongoing increase in life expectancy [76] and subsequent prolonged age-related bone loss, an increasing number of individuals are likely to reach the diagnostic threshold for osteoporosis in later life. Moreover, as the world's population aged 60 and 80 years and over is estimated to increase three and seven fold by 2100, respectively [76], osteoporosis will become an increasingly bigger health burden worldwide. Currently, strategies focusing on the prevention of the condition are scarce, and thus further understanding of its risk factors is urgently needed for the development of more strategies which could potentially minimise the increasing health burden associated with osteoporosis in the near future.

1.2.7 Risk factors for osteoporosis

Although still not fully understood, it has previously been established that the underlying mechanisms of osteoporosis are multifactorial with genetic, biological and environmental risk factors all contributing to the development of the condition. The importance of genetics as a risk factor for osteoporosis was highlighted by findings of a UK study that reported the heritability of lumbar spine and femoral neck BMD of around 78% and 84%, respectively [77]. Moreover, recent experimental data has identified a number of single nucleotide polymorphisms that have been associated with a 20-30% increased risk for osteoporosis [78]. Currently well-established biological factors associated with an increased risk of developing osteoporosis include increasing age [30, 31], being female [27], undergoing the menopausal transition or being post-menopausal [79, 80], having a family history of osteoporosis [81, 82] and being Caucasian [83]. Similarly, a number of lifestyle factors have also been shown to increase the risk of osteoporosis and those include low body mass index (BMI) [27, 84, 85], current or former smoking [27, 79], low physical activity [79, 86] and the use of some medications including anti-diabetic drugs [87] and steroids [88]. Dietary factors associated with an increased osteoporosis risk include low dietary intakes of calcium, protein and fruit and vegetables [89-91]. Other dietary factors, including high alcohol and caffeine intake, may also contribute to an increased osteoporosis risk, although current evidence is not convincing [92-95].

In contrast, there are a number of beneficial factors which may delay the onset of osteoporosis, possibly through decreasing bone resorption and enhancing bone formation. Examples of these

include hormone replacement therapy (HRT) [27], weight-bearing exercise [96] and the use of dietary supplements including calcium and vitamin D [97-99].

1.3 Osteoporotic fractures

1.3.1 Definition and underlying mechanisms

The thin and porous bones associated with the progression of osteoporosis may eventually be unable to withstand even minimal impact, often during routine daily activities, causing them to fracture. Osteoporotic fractures are the clinical consequences of osteoporosis. They differ from non-osteoporotic fractures in that the bone collapses, i.e. it shatters and falls in on itself, causing pain, fragility and immobilisation for the individual. They may affect any bone, but the most common sites include the hip, the spine and the wrists.

The underlying mechanisms of fractures are complex, but are predominantly linked to the loss of bone in osteoporosis. For example, it has been shown that a 1 SD reduction in bone density from the mean value for an age-specific population was associated with an approximate 2-3 fold increase in long-term fracture risk [21, 100]. Moreover, the recent development of FRAX™ by the WHO, a fracture risk assessment tool designed to determine an individual's 10-year probability of a hip or major osteoporotic fracture, showed that fracture risk prediction was enhanced when BMD was included in the prediction model [101]. It has been suggested that the predictive ability of bone density for the risk of fracture is comparable to that of a 1 SD increase in diastolic blood pressure for stroke risk for a similar age-specific population [21].

1.3.2 The prevalence of osteoporotic fractures

Osteoporotic fractures are a global health issue with an annual prevalence of 8.9 million fractures worldwide [102]. As with osteoporosis, the risk for fracture is much higher in women compared to men. For example, it is estimated that in Britain, one in two women and one in five men over the age of 50 years will most likely suffer a fracture as a result of osteoporosis [103]. At specific ages, the risk even outweighs that of other chronic diseases, with women aged 50 years having a greater lifetime risk of osteoporotic fractures than the risk of developing breast cancer and cardiovascular disease [63]. In the UK, there are more than 60,000 hip fractures, 50,000 wrist fractures and, most commonly, 120,000 fractures of the spine each year [103-105]. Hip fractures alone are estimated to account for an annual financial burden of approximately £2.3 billion to the British National Health Service (NHS) for the subsequent hospital and social care [75]. Hence, it is the high prevalence as well as the significant personal and economic burden [106, 107] of those osteoporotic fractures that makes osteoporosis such an important health issue. With the continuing increase in the number of older people and their higher life expectancy, fractures

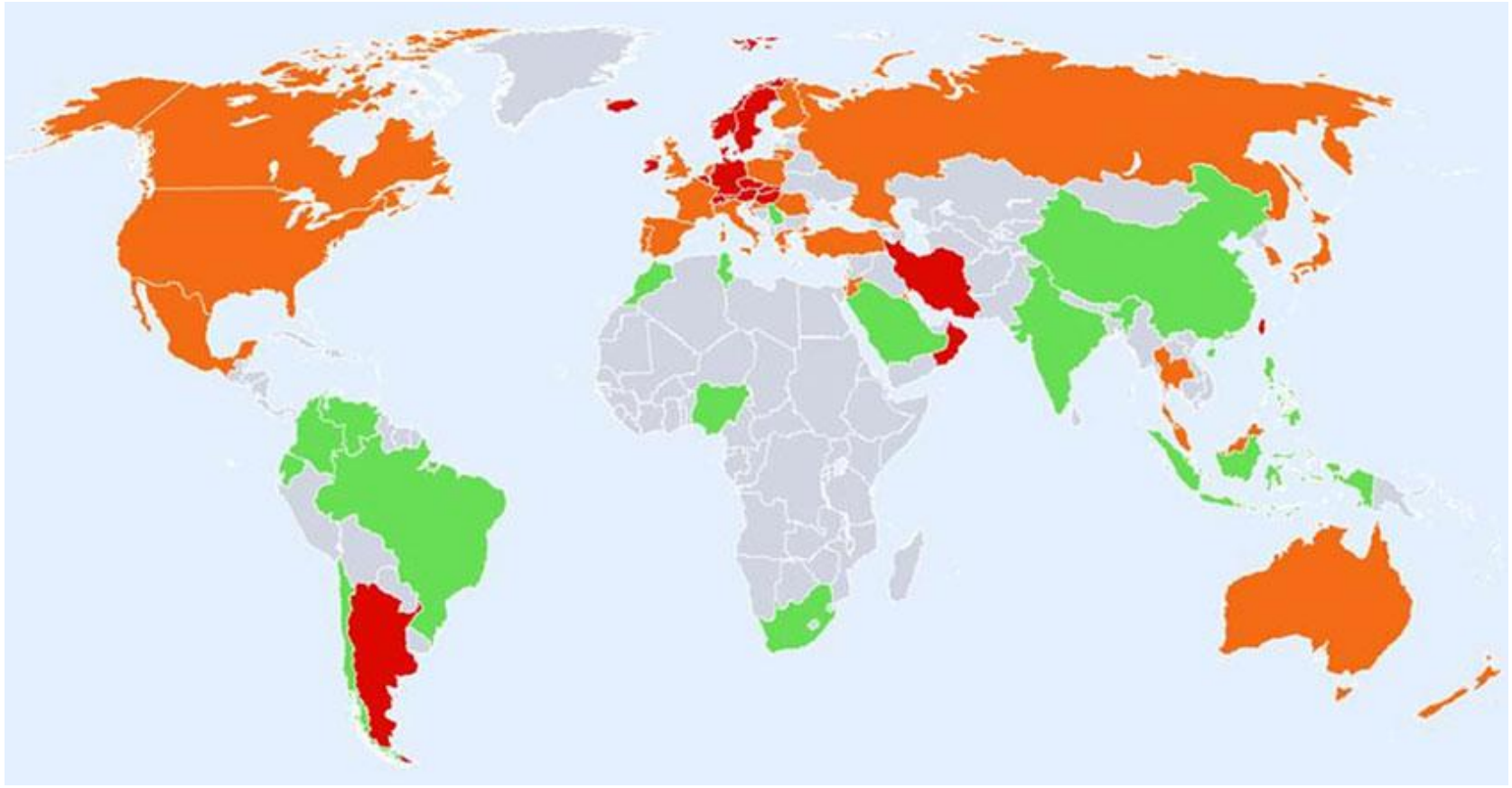
along with osteoporosis will become an even greater economic health burden over the next few decades.

Fracture rates differ by geographical region by up to 10 fold **(Figure 1.4)** [108]. For example, the age-standardised hip fracture incidence in men and women combined is estimated to be 55/100,000 in Ecuador, 119/100,000 in Indonesia, 172/100,000 in Romania and 250/100,000 in the UK [108]. Differences in lifestyle behaviour, including the composition of diet, may partly be responsible for the discrepancies between countries.

1.3.3 Risk factors for fractures

With fractures being the clinical endpoint of osteoporosis, risk factors associated with developing the latter are thus equally relevant to fracture risk. Those include genetic factors [78], increasing age [109, 110], being female [103, 111], menopausal status and years since menopause [79], being Caucasian [83], low BMI [112], smoking [113] , low physical activity [109], a number of medications including steroids [114] and dietary factors including low calcium intakes [115]. Additional factors which are thought to increase fracture risk include a family history of hip fracture [116], a prior fragility fracture [117], a high prevalence of falls [118], as well as high dietary alcohol intakes [119]. In contrast to these risk-increasing factors, beneficial factors which have been associated with a decrease in fracture risk include HRT [79] and the use of dietary supplements including calcium [120] and vitamin D [121].

Figure 1.4: The global geographic distribution of hip fracture risk for men and women.



Annual hip fracture incidence by country: >250/100,000 (red), 150-250/100,000 (orange), <150/100,000 (green), no data available (grey). From Kanis et al. 2012 [108].

1.4 Diet, osteoporosis and fractures

Currently, the underlying pathology of osteoporosis and osteoporotic fractures is not fully understood; thus the understanding of potentially preventative mechanisms of the condition is limited. As previously discussed, a number of risk factors may contribute to the development of osteoporosis and fractures by influencing the underlying pathology of the condition. Lifestyle factors including diet are modifiable in contrast to most biological factors (e.g. increasing age, being female), and their modifiability may be a useful approach in preventing osteoporosis and subsequent fracture development. For example, it might be possible to reduce age-related bone loss or modify changes in BMD occurring as a result of other non-modifiable risk factors, such as being female, through the use of diet. There are a number of dietary factors thought to be important for the prevention of osteoporosis and fractures, including calcium with and without vitamin D [120, 122], total protein [123] and plant-based foods such as fruits and vegetables [90, 124]. However, a number of other micronutrients, including vitamin K_1, vitamin C and iron may have equally important roles in bone health through specific underlying mechanisms [7-11]. The potential importance of these modifiable dietary factors in reducing osteoporosis and fracture risk will be discussed in this section.

1.4.1 Calcium and vitamin D

To date, the main focus of research relating to diet, osteoporosis and fracture prevention has been on calcium and vitamin D sufficiency. This is because 99% of the body's calcium is found in bone and teeth, providing strength and stability; and vitamin D is involved in maintaining calcium homeostasis [125]. Dietary calcium intakes of >500 mg/d have previously been associated with higher BMD [91, 126] and lower fracture risk [127]; whereas intakes of <700 mg/d were associated with an increased risk of hip and any-type fracture [115]. Furthermore, there is evidence from RCTs and observational studies that the use of calcium supplements reduces bone loss, particularly at low calcium baseline levels [99], and may decrease fracture risk by 25-70% [120]. Vitamin D sufficiency has primarily been studied using supplemental intakes rather than intakes from foods, as the body's main source is from sun exposure and much less from foods [128]. The use of 400 IU/d of vitamin D supplements has been shown to increase BMD of the femoral neck [97] and reduce bone loss at the spine [98]. Moreover, a meta-analysis of double blind RCTs showed that supplementation with 700-800 IU/d, but not 400 IU/d, for 12-60 months was associated with a reduction in hip and any non-vertebral fracture risk by 26% and 23%, respectively [121].

1.4.2 Protein

Protein has also been a major nutrient of interest for bone due to its potentially negative effects at low and high intakes. Low protein intakes may result in decreased intestinal calcium absorption and increased levels of parathyroid hormone, leading to the release of calcium from bone [89]. In contrast, higher protein intakes have previously been associated with increased urinary calcium excretion and subsequent negative calcium balance [129]. A recent systematic review and meta-analysis showed positive effects of protein supplementation on spine BMD (weighted mean difference 0.02, 95%CI 0.00-0.04; *P*=0.04), although these beneficial effects were not found in the long-term with hip fracture risk (highest *vs.* lowest quantile: RR 0.75, 95%CI 0.47-1.21; *P*=0.24) [6]. There is also inconsistent evidence for the effects of different protein sources on bone, with the overall consensus being that animal protein is neither more beneficial nor detrimental to bone than is plant protein [89, 130]. In addition to the overall effects of total protein intake on bone, individual amino acids may have differing underlying mechanisms so that some may be more relevant to overall bone health than others. For example, the amino acids proline and lysine are required for adequate collagen formation and maintenance [9], and thus may potentially be important for bone health.

1.4.3 Fruit and vegetables, potassium and magnesium

Findings from a systematic review showed that the link between fruit and vegetable consumption and markers of bone turnover, BMD and fractures is not yet well defined [5]. However, a higher dietary intake of fruit and vegetables has previously been associated with greater BMD and reduced BMD loss in a number of observational studies [90, 131-133]. Moreover, in a recent prospective study of 75,591 Swedish men and women aged 45-83 years, participants with ≤ one serving of fruit and vegetable per day had significantly higher hip fracture risk compared to those subjects consuming five servings per day (HR 1.88, 95%CI 1.53-2.32 and HR 1.35, 95%CI 1.21-1.58, respectively) [134]. Although the potentially underlying mechanisms are still unclear, it has been suggested that fruit and vegetables may exert beneficial effects on bone health by providing a number of compounds which are involved in bone metabolism [135]. For example, fruit and vegetables contain micronutrients including potassium, magnesium and vitamin C, which are essential to the synthesis of bone tissue [136] and which may lower the acid load of the diet, thus reducing the necessity for bone to act as an alkaline buffer [90, 137]. Moreover, phytochemicals, antioxidants and other bioactive compounds may mediate bone metabolism by reducing inflammation and oxidative stress [138-140].

Both potassium and magnesium have been extensively studied as individual explanatory nutrients for the beneficial associations between fruit and vegetable intakes and bone health [90, 133, 141-144]. Fruits and vegetables have very high potassium contents [145], and potassium is known

to be important for maintaining both renal calcium retention and the tightly-controlled acid-base balance (pH 7.35-7.45) [90, 141]. In previous cross-sectional studies, higher potassium intakes were significantly associated with greater BMD at multiple bone sites in both men and women [90, 133, 142]. Moreover, the results from a recent meta-analysis of 14 studies showed that the potassium bicarbonate and potassium citrate supplementation was associated with significantly lower urinary calcium excretion and the bone resorption marker NTx, although there were no effects on BMD and bone formation markers [146]. Magnesium is predominantly found in green leafy vegetables [145] and may exert beneficial effects on bone via its involvement in regulating calcium homeostasis and its incorporation into the bone matrix and subsequent importance for the structural integrity of bone [90, 124, 143, 147, 148]. A number of observational studies have shown positive associations between magnesium intake and BMD at different sites in both men and women [90, 133, 143, 144, 149]. Moreover, short-term beneficial effects of magnesium supplementation in the form of magnesium citrate (1830 mg/d) were reported in an intervention study of 20 postmenopausal women, where markers of bone turnover were suppressed following 30 days of treatment [147]. Furthermore, findings from an RCT (*n*=54) showed that the supplementation with magnesium hydroxide (250-750 mg/d) for 6-24 months significantly increased trabecular BMD in postmenopausal women with osteoporosis compared to osteoporotic controls [148].

1.4.4 Vitamin K_1

Vitamin K_1 is predominantly found in fruit and vegetables, yet its role in bone health has been less well studied. Vitamin K_1 acts as a cofactor in the γ-carboxylation of osteocalcin, the most abundant non-collagenous protein in bone, which is crucial for its ability to bind calcium [11]. As previously discussed, calcium is an important structural element to bone, providing both strength and stability [125]. Vitamin K_1 has also been associated with the down-regulation of IL-6 expression in osteoclastogenesis, and subsequent reduced bone-related inflammation [150, 151]. There is evidence from RCTs in women to show that vitamin K_1 supplementation of 100 µg/d for 12 months resulted in 1.1-1.35% higher BMD at multiple sites compared to baseline [152]; and 1000 µg/d for three years were associated with 1.7% reduced BMD loss compared to the control group [153]. In previous epidemiological studies, higher dietary vitamin K_1 intakes were associated with higher BMD *(no effect sizes shown)* [154, 155]; and every 100 µg/d increment in intake was associated with an 0.96 dB/MHz increase in BUA and a 1.13 m/s increase in SOS [156]. Moreover, prospective and longitudinal studies have almost consistently shown that higher dietary vitamin K_1 intakes are associated with a lower risk for hip fracture [157-159]. For example, hip fracture risk was up to 65% lower in men and women with median dietary vitamin K_1 intakes of 254 µg/d compared to 56 µg/d after 7-years of follow-up [158]; and every 10 µg/d increment in dietary vitamin K_1 intake was significantly associated with a 2% reduction in hip fracture risk [157]. However, to date, epidemiological evidence in men for a potential beneficial role of vitamin K_1 in

bone health is scarce [155-158]. Moreover, data from British cohorts is limited to only one previous study of early post-menopausal women [154], despite previously reported differences in dietary vitamin K_1 intakes between populations [158-160].

1.4.5 Vitamin C

Vitamin C is exclusively found in fruit and vegetables, but evidence for a potential role in bone health is limited. Vitamin C is crucial for bone collagen synthesis and maintenance via acting as a cofactor in the hydroxylation of proline and lysine residues within collagen fibres [9], thereby contributing to the adequate formation of collagen cross-links and subsequent stronger collagen [136]. In bone, collagen is predominantly present in around 98% of the organic phase of bone [13]. Additionally, vitamin C has been suggested to be involved in osteoclastogenesis and osteoblastogenesis, potentially via mediating RANKL expression and PPAR-γ expression, respectively [161-164]. In previous epidemiological studies, higher intakes of dietary vitamin C were associated with 3-5% higher BMD in women [133]; and women who reported the use of vitamin C supplements (70-5000 mg/d) compared to non-users have been shown to have 4% higher BMD [165]. Moreover, in prospective studies, BMD loss after 2-5 years of follow-up was 54% reduced in men and women with higher compared to lower dietary intakes of vitamin C [166]; and the use of vitamin C supplements (mean: 260 mg/d compared to 0 mg/d) was associated with a 44% reduction in hip fracture risk after 15-17 years of follow-up [167]. However, my recent review of the literature on vitamin C and bone health [168] highlighted that data on plasma or serum concentrations is scarce [166, 169] which have the benefit of eliminating human recall error in contrast to estimations of dietary intake [170]. Evidence of a role of vitamin C in bone health is particularly scarce for men despite fractures becoming an increasing health problem in both sexes [171]. Furthermore, there is only limited data from British populations and of those studies, all had studied less than 1,000 participants [124, 133, 166]. Finally, studies investigating vitamin C intake with fracture risk as the clinical endpoint of osteoporosis have currently only been reported from two US studies [167, 169].

1.4.6 Iron

Iron is also a crucial cofactor in hydroxylation reactions in bone collagen synthesis, where it undergoes a cyclic oxidation and reduction [9]. Moreover, iron is involved in converting vitamin D into its active form (1,25-dihydroxycholecalciferol) via acting as a cofactor to the reaction-specific enzyme 25-hydroxycholecalciferol 1-hydroxylase [172]. Vitamin D is an important mediator of the homeostasis of calcium, and the latter is an important structural element to bone [125]. A number of animal studies have previously reported lower bone mass and mechanical strength in iron deficient and severely iron deficient rats [173-176], as well as decreased

bone formation and increased bone resorption [174, 177]. However, evidence from human studies is limited. Although evidence from RCTs has shown that the rate of bone resorption was significantly higher in anaemic participants compared to healthy controls [178], published intervention studies were small and were only conducted in women [178-180]. In observational studies, dietary iron intake was significantly associated with greater BMD at multiple sites in women, for example 4-14% higher BMD for the highest *vs.* lowest intake [181] or β 0.214-0.426 g/cm^3 ($P \leq 0.05$) [142]. However, these studies had small sample sizes (n=242-244) and epidemiological investigations of iron intake in men are completely lacking. There is also some evidence for a beneficial role of higher iron status in bone health [182-184]. For example, higher serum ferritin levels in men were significantly associated with higher BMD at multiple sites (β 0.008-0.018 g/cm^2, $P \leq 0.049$) [182]. To date, the potential role for iron in the prevention of fractures has only been investigated in one prospective study, which reported a five-fold increase in fracture risk with higher iron status (OR 5.27, 95%CI 1.12-24.94) [185], possibly due to the short follow-up period of three years and the known detrimental effects of increasing age on bone health [30, 31]. Finally, there is data from only one British study of 32 women, which showed that higher dietary iron intakes were significantly associated with reduced spinal BMD loss (β 0.141 g/cm/year, $P<0.0001$) [186]; and thus data from British populations is needed.

To date, the role of iron in bone health has only been studied independent of the food source [142, 181, 186]. However, the dietary intake of iron provides no information on its bioavailability which differs between its two different forms: i) haem iron, a derivative of haemoglobin and myoglobin found in animal-based products, and ii) non-haem iron, which is present as iron salts in both animal- and plant-based foods [187]. The two forms differ in their level of intestinal absorption, with haem iron being more efficiently absorbed than non-haem iron (15-40% *vs.* 1-15%) [188-192]. In our diet, haem iron is the greater contributor towards the body's iron pool resulting from its higher level of absorption, despite non-haem iron making out a greater percentage of total dietary iron intake [193]. It is unclear whether or not iron from plant- and animal-based sources may vary in their underlying mechanisms in collagen synthesis [9] and vitamin D synthesis [172], and thus the consequences of this for bone health are not known. To the best of my knowledge, no studies have explored potential differences in iron sources in their role in bone health, therefore such investigations are warranted and are completely novel.

1.5 Deriving the dietary intake of individuals and populations

The overarching aim of nutritional research is to record daily actual intake, also referred to as true intake, i.e. the exact types and quantities of foods and drinks consumed at any one time at the individual or population level [194]. There are a range of methods used in nutritional epidemiology to quantitatively assess food and nutrient intakes on the individual and population

level [195]. They mainly differ in the type of intake they assess (habitual or recent intake), the accuracy of intake assessment and their practicability. The most commonly used methods are the 7-day diet diary (7dDD), food frequency questionnaire (FFQ), 24-hour recall (24hR), 16-day weighed record as well as biological markers of dietary intake. However, as studies are undertaken in humans and are subject to practical and ethical constraints, it is generally accepted that no measure of dietary assessment can completely capture true intake [194]. The following section will give a more detailed account of the different types of dietary assessment methods and their strengths and limitations.

Dietary records such as the weighed record and the 7dDD require individuals to record all foods and beverages as they are being consumed, as well as their amounts, for a specified period of time, usually three to seven days [195]. Weighed records and 7dDDs differ in that the former requires the participant to weigh each food item using scales, whereas the latter is based on portion size estimates. Dietary records provide a relatively accurate indication of usual intake due to the nature of keeping a diary. Moreover, as food and drinks are recorded as they are being consumed, the reliance on long-term memory is not an issue. However, dietary records, especially weighed records, are expensive and their practicability for studies with large sample sizes is small due to the nature of recording and processing diaries. Moreover, they are prone to under-reporting resulting from behaviour modification, meaning that participants may i) alter their habitual food intake as a result of the recording situation or ii) chose not to record food items despite having consumed them.

FFQs are designed to estimate habitual food intake. They contain a checklist of limited foods and beverages with a frequency category and sometimes also a quantity response section which estimate how often individuals consume each item over a specified period of time, usually the past 12 months [195]. They are relatively inexpensive and quick to administer, and thus are more practical for use in larger population studies, where the ranking of participants into categories is preferred. However, FFQs are less precise in estimating dietary intakes than 7dDDs as they are restricted to a chosen number of listed food items, do not derive detailed information on food preparation methods and rely heavily on an individual's long-term memory [196]. Moreover, over-estimation of dietary intakes is a common issue with FFQs and is directly related to the length of the FFQ food list [197]. FFQs may overestimate dietary intakes proportionally for the whole study population, although the extent to which this may affect the association between FFQ and disease needs to be clarified.

The 24hR provides a quick and detailed description of an individual's most recent dietary intake. They are a popular choice amongst studies with larger sample sizes due to their low cost and quick administration. The 24hR is usually conducted by an interviewer and requires individuals to report all foods and beverages and their quantities consumed in the past day [195]. It is thus prone to inaccurate dietary intake reporting by the individual due to the interview

situation. Moreover, the assessment relies on short-term memory recall and cannot account for episodic foods as well as day to day and seasonal variation in dietary intake. However, the 24hR is less likely to be affected by behaviour modification resulting in changes in usual food intake as it is completed the day after the food has been consumed.

Biological markers, that are sensitive to dietary intakes, are typically measured in blood or urine. They are useful for validating other dietary assessment methods and for determining changes in dietary intake behaviour over a specified period of time as they eliminate reporting bias [(195)]. However, it is generally accepted that biomarkers are not a true reflection of dietary intake due to homeostatic mechanisms influencing biological processes including nutrient absorption and excretion. Thus, a low level of agreement between nutrient intake and biomarker does not necessarily infer inaccuracy of the dietary measure. Moreover, biological assessments are time-consuming, often invasive to the participant and expensive to perform, and are thus not commonly used in studies with large sample sizes [(198)]. They also tend to relate to only a very limited range of nutrients such as urinary nitrogen for protein intake and plasma or serum vitamin C for vitamin C intake.

In summary, written dietary assessment methods and biomarkers are inaccurate estimates of the habitual dietary intake. The choice of assessment usually depends on the sample size of the study population, the practicality of the assessment and evaluation of intake and the subsequent costs involved. Moreover, the methods differ in their accuracy in assessing specific nutrients and may be used based on their suitability in estimating the nutrient of choice.

1.5.1 Measurement error in dietary assessments

In nutritional epidemiology, measurement error refers to the difference between the dietary intake that was recorded and the true intake. The presence of measurement error in all nutritional epidemiological studies is an important issue as it reduces the statistical power to detect diet-disease associations [(198-200)]. To date, dietary assessment methods, which are completely free from measurement error, have not been established. Currently available methods are self-reported and are based on a compromise between speed and accuracy of performing the dietary assessment and evaluating food intake [(195)]. Consequently, recorded dietary intake is an approximate estimate rather than a true reflection of intake. Moreover, food intake varies daily, weekly and by season [(194)]. As it is impractical to record food consumption continuously, an average habitual intake must be derived over a specified number of days, aiming to reflect true intake most accurately. The assessment of dietary intake introduces different levels of measurement error which may relate to the recording of food intake, the specific assessment method used, as well as data entry and analysis programmes. For example, the recording situation in itself often makes participants feel exposed and judged, resulting in behaviour modification and subsequent misreporting of habitual dietary intake, usually towards

a healthier intake during the period of assessment [195]. In concordance with this, underreporting (particularly of snack foods) and sometimes overreporting (of fruit and vegetables) are well-known issues of dietary assessment [201-204]. Measurement error may also arise from the specific dietary assessment techniques which differ in the type of intake they assess (habitual or recent intake), their accuracy and their practicability, the latter of which may affect the level of commitment by the individual [195]. Some methods use trained interviewers to perform the assessment which introduces additional interviewer bias. Once the assessment has been completed, the data entry may also be subject to error, for example resulting from data entry errors or subjective assumptions of food intake and portion sizes by the investigator. Moreover, the use of nutrient analysis programmes, which convert the foods eaten into quantities of specific nutrient intakes, introduce error depending on how these food-to-nutrient conversions were derived and the size of the food and portion size databases [195]. The extent to which measurement error may be present in nutritional epidemiological studies is unknown, but is likely to vary by study, as it is dependent on a number of factors including the type of dietary assessment, the data entry procedures and the derivation of food-to-nutrient conversions.

To date, statistical techniques, which completely eliminate all measurement error, do not exist. Previous studies aiming to address the methodological issues of dietary assessments have suggested that combining the data for food intake and serum status in a population may somewhat reduce the measurement error in the subsequent diet-disease associations [205, 206]. This is because dietary intake does not directly translate into nutrient status in the body, as the latter accounts for individual differences in a number of biological processes including absorption, metabolism and excretion [207]. To the best of my knowledge, this approach has not previously been used in epidemiological studies investigating diet and bone health associations. However, it could provide a new strategy for improving the methodology of such studies in the future, thereby determining potentially more accurate associations between diet and bone health.

1.6 Research gaps

To date, a number of nutrients including calcium, vitamin D, total protein, potassium and magnesium have been extensively studied in relation to osteoporosis and fracture risk. However, despite potentially important underlying mechanisms, there has been limited data on vitamin K_1, vitamin C and iron. Further population-based studies are required as current evidence is scarce and lacks consistency. Moreover, potential associations between different dietary sources of iron with bone health are completely lacking. These investigations are crucial for establishing optimal nutrient intakes and for developing more specific dietary strategies regarding osteoporosis and fracture prevention.

Despite nutritional research aiming to record true food intake, it is commonly accepted that there are no measurements of dietary intake which are free from measurement error. Previous validation studies have suggested that measurement error of diet-disease associations may be reduced by combining the food intake with nutrient biomarkers within a population. Thus, diet-disease associations, which are based on a combined value for food intake and nutrient status, may be more accurate than those relying on separate measurements. However, this approach has not previously been used in epidemiological studies on diet and bone health, but this may be an important strategy for improving the methodology of such future studies.

The present thesis will address these research gaps by investigating associations between habitual dietary intakes and nutrient status in blood with heel ultrasound and fracture risk in a population-based study of older men and women. The thesis will also contribute to the understanding of how different dietary assessment methods and biomarkers impact on epidemiological associations between diet and bone health.

1.7 Thesis aims and hypotheses

The overall aim of this thesis was to investigate and compare the associations between habitual dietary intakes and nutrient status in blood with heel ultrasound and fracture risk in older British men and women of the Norfolk-based European Prospective Investigation into Cancer and Nutrition (EPIC-Norfolk) cohort. The EPIC-Norfolk cohort is an ongoing prospective study which is part of the larger EPIC study (over 500,000 participants in total) and which examines behavioural markers such as dietary intake and multiple disease endpoints including osteoporosis and fractures in British men and women based in Norfolk. A more detailed account of this cohort follows in Chapter 2 (page 40). It was hypothesised that higher dietary intakes as well as biological markers of nutrient status are i) positively associated with heel ultrasound and ii) inversely associated with the risk of fracture. Additionally, it was hypothesised that the combination of dietary intake with markers of nutrient status in blood would strengthen these associations.

1.8 Objectives

i) To examine potential cross-sectional associations between a number of micronutrients (vitamin K_1, vitamin C and iron) as well as nutrient status levels in blood (plasma vitamin C and serum ferritin) with measures of heel ultrasound.

ii) To investigate whether these micronutrient intakes and nutrient status are prospectively associated with fracture risk at three common fracture sites (hip, spine and wrist).

iii) To contribute to the development of an iron database of foods within the EPIC-Norfolk food database, which identifies the animal and plant source contributions of iron, before investigating potential cross-sectional and prospective associations between animal and plant-based iron intake with measures of heel ultrasound and fracture risk, respectively.

iv) To investigate whether combining different dietary assessment methods may strengthen diet-disease associations compared to using single measurements at the example of vitamin C and its relationship with bone health.

CHAPTER 2

METHODOLOGY

2.1 Introduction

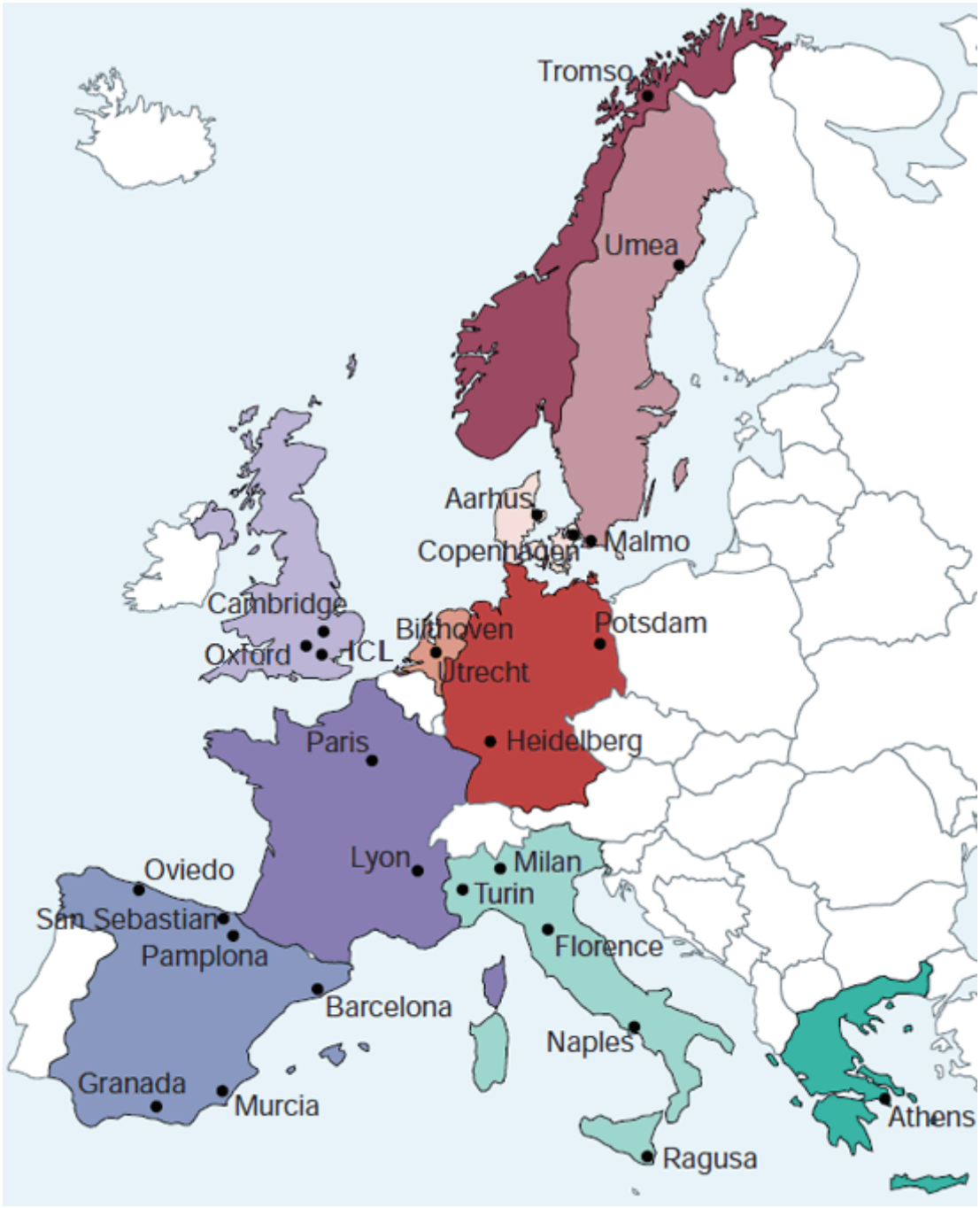
The present observational investigations used data previously collected from the EPIC-Norfolk study of 25,639 participants aged 39-79 years at baseline. The main analyses consisted of a cross-sectional study that assessed associations between bone density, using heel ultrasound measurements, and nutrient intake and nutrient status in 2341 participants of a random sub-cohort (*n*=4000) of EPIC-Norfolk. Secondly, a prospective study of fracture risk with a median follow-up of 12.6 years was undertaken in a case-cohort subset of EPIC-Norfolk of 5319 participants to investigate potential associations with nutrient intakes and status. The data from both studies were also used to investigate whether the addition of a biomarker to a dietary intake estimate would improve the detection and strength of the diet-disease association, using the association between vitamin C intake and status with heel ultrasound and fracture risk as an example.

2.2 The EPIC-Norfolk Cohort

EPIC is a prospective cohort study of more than half a million participants (521,000) initiated in 1989 with collaborations set up between 23 centres in 10 European countries to establish the relationship between diet and the risk of developing common cancers **(Figure 2.1)**. EPIC-Norfolk is one of the UK sub-cohorts of this prospective investigation based in Cambridge and has additionally defined causes of disability and death in mid-aged and older people. Recruitment was undertaken in 35 general practices in inner-city, sub-urban and rural areas of Norfolk. A total of 25,639 mainly Caucasian men and women aged 39-79 years attended the first health check between 1993 and 1997. The data collection included the following:

i) a health and lifestyle questionnaire with questions on smoking, alcohol consumption, exercise, socio-economic status, social class, occupational history, medical status, family history of main diseases and reproductive history (for women);
ii) a questionnaire on major depressive disorder and generalised anxiety disorder;
iii) a health check at baseline with examinations and samples taken for respiratory function, anthropometry, blood pressure, urine testing, and with additional examinations of body composition and quantitative ultrasound of the heel bone at follow-up every 18 months;
iv) and dietary assessments including a food frequency questionnaire (FFQ), a 24-h recall and a 7-day diet diary (7dDD).

Figure 2.1: Centres of the EPIC cohort study.



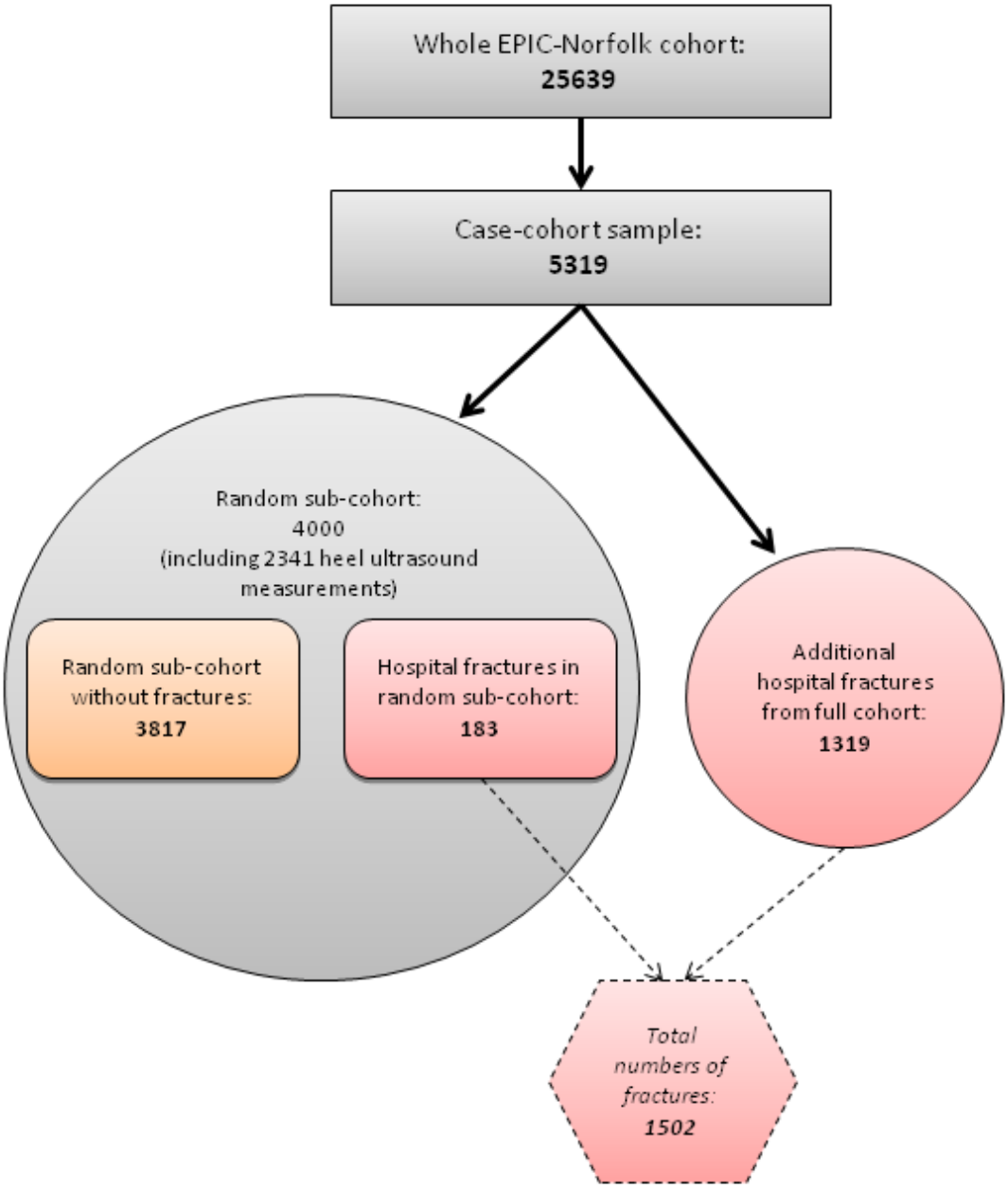
The map includes the coordinating centre of EPIC, Imperial College London (ICL).

EPIC-Norfolk participants were invited to attend follow-up clinic visits in 1997-2000 and 2006-2011. The whole cohort is still being followed-up to date for different health points including cancer incidence through cancer registration, mortality by cause through death certification, and by means of posted questionnaires and health record linkage. All participants gave an informed consent at the beginning of the study. The study was approved by the Norwich District Health Authority ethics committee and was conducted according to the Declaration of Helsinki.

2.3 The Dataset

For the present study, data from the whole cohort (n=25,639) was unavailable for analysis. The cross-sectional study of heel ultrasound was based on a random sub-cohort of 4000 participants, representative of those participants who had attended the first health check between 1993 and 1997 **(Figure 2.2)**. The smaller representative dataset was used for all analyses to ensure that no selection bias was introduced into the present study because data entry as well as data cleaning and processing were not yet complete at the time of writing. The prospective investigations of fracture risk used data collected up to 31st March 2009 and were based on a case-cohort design of the same subset of 4000 participants and a set of 1502 participants who had experienced a fracture. Accounting for the overlap between the sub-cohort and the fracture cases, the total number of participants of the prospective study was 5319 men and women, representing 21% of the full cohort.

Figure 2.2: The structure of the present random sub-cohort and the case-cohort sample from the EPIC-Norfolk study.



2.3.1 Non-dietary exposure assessment

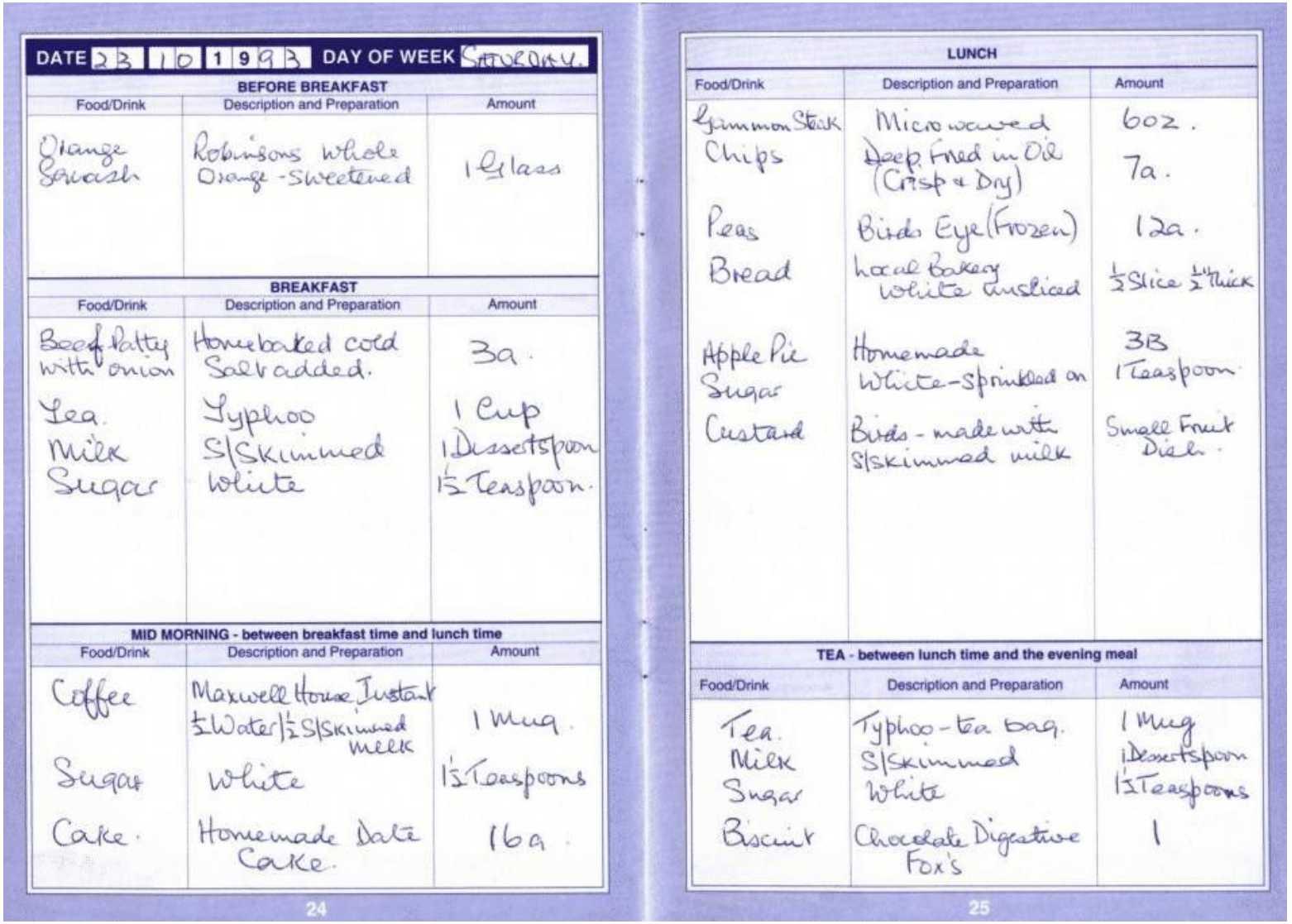
Age, family history of osteoporosis, smoking (current, former and never), physical activity [208] (active, moderately active, moderately inactive and inactive), menopausal status in women (premenopausal, early perimenopausal [<1 year], late perimenopausal [1-5 years] and postmenopausal [>5 years]), HRT use in women (current, former and never) and medication use including steroid medication were derived from a health and lifestyle questionnaire administered at each health check [209]. Weight and height, measured to the nearest 0.2 kg with a digital scale (Salter) and to the nearest 0.1 cm with a free-standing stadiometer, respectively, were taken on participants dressed in light clothing and without shoes. BMI was calculated as weight in kilograms divided by height in meters squared.

2.3.2 Dietary exposure assessment

EPIC-Norfolk used several different dietary assessment methods to record average food intake at baseline. Firstly, a semi-quantitative food frequency questionnaire (FFQ) and a self-reported 24-h recall (24hR) were both completed before the initial health check. Secondly, a 7-day diet diary (7dDD) was completed immediately after this health check [209]. The latter was an A5 booklet [210] **(Figure 2.3)** which was based on the diary used in the National Survey of Health and Development [211]. It contained seventeen colour-print photographs of foods to aid portion size estimation, four pages for recording the foods and drinks consumed each day, a recipe notation, a checklist of commonly consumed foods as well as a short questionnaire regarding the types of milk, bread and spread consumed. The first day of the 7dDD was an interviewed 24-h recall conducted on-site by a nurse according to a standardised protocol [212]. At home, participants were requested to report all foods and drinks consumed for the remaining 6 days.

Previous validation studies on this cohort have shown that the estimated 7dDDs were most comparable to weighed food records for the majority of nutrients [202, 204]. For example, vitamin C intake estimated from a weighed food record correlated better with intake measured from the 7dDD (r=0.70) compared to the FFQ and the self-reported 24-h recall (both r=0.54). The conversion of participant reported text into quantitative food data was previously undertaken with the in-house data-entry program Data into Nutrients for Epidemiological Research (DINER), which is based on more than 11,000 food items and almost 600 portions [212]. Responses regarding supplement intake were entered into the vitamin and mineral supplement database (ViMiS) [213]. The estimation of dietary vitamin K_1 intake from 7dDDs was based on a previously published database [214]. However, as the food content of vitamin K_1 was still largely incomplete, the database had been developed further by EPIC-Norfolk nutritionists who added predominantly British food items [160, 215].

Figure 2.3: Example of one day of a 7dDD used in the EPIC-Norfolk study.



DATE 23 10 1993 DAY OF WEEK SATURDAY.

BEFORE BREAKFAST

Food/Drink	Description and Preparation	Amount
Orange Squash	Robinsons Whole Orange - Sweetened	1 Glass

BREAKFAST

Food/Drink	Description and Preparation	Amount
Beef Patty with onion	Homebaked cold Salt added.	3a.
Tea.	Typhoo	1 Cup
Milk	S/Skimmed	1 Dessertspoon
Sugar	White	1½ Teaspoon.

MID MORNING - between breakfast time and lunch time

Food/Drink	Description and Preparation	Amount
Coffee	Maxwell House Instant ½ Water/½ S/Skimmed Milk	1 Mug.
Sugar	White	1½ Teaspoons
Cake.	Homemade Date Cake.	16a.

24

LUNCH

Food/Drink	Description and Preparation	Amount
Gammon Steak	Microwaved	6oz.
Chips	Deep Fried in Oil (Crisp & Dry)	7a.
Peas	Birds Eye (Frozen)	12a.
Bread	Local Bakery White Unsliced	½ Slice ¼ Thick
Apple Pie	Homemade	3B
Sugar	White - Sprinkled on	1 Teaspoon
Custard	Birds - made with S/Skimmed milk	Small Fruit Dish.

TEA - between lunch time and the evening meal

Food/Drink	Description and Preparation	Amount
Tea.	Typhoo - tea bag.	1 Mug
Milk	S/Skimmed	1 Dessertspoon
Sugar	White	1½ Teaspoons
Biscuit	Chocolate Digestive Fox's	1

25

Nutrient data from these previously completed 7dDDs were available for the present cross-sectional and prospective studies, hence providing relatively accurate estimates of nutrient intakes. For the sub-study, which combined intake and biomarker measurements of vitamin C to investigate the association with heel ultrasound and fracture risk, data for vitamin C intake estimated from both a 7dDD and a FFQ were available for analysis, in addition to plasma vitamin C concentrations.

2.3.3 Biological markers of nutrient status

2.3.3.1 Plasma vitamin C

Plasma vitamin C, as an indicator of vitamin C status, was previously measured in participants from non-fasting blood samples taken at baseline. Blood was drawn into citrate bottles and refrigerated overnight at 4-7°C in dark boxes before being centrifuged at 2100g for 15 minutes the following day [(216)]. Plasma was stored at -70°C following its stabilization with a standardized volume of metaphosphoric acid. Plasma vitamin C concentration was estimated within one week of blood sampling using a fluorometric assay [(217)]. The coefficient of variation was 6.2% and 2.7% at the lower and upper end of the range, respectively.

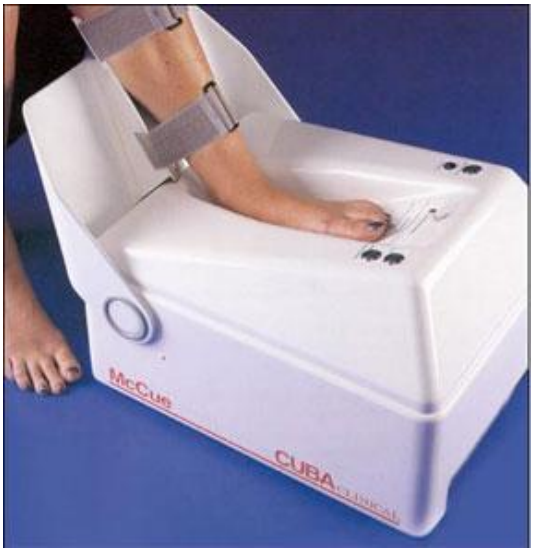
2.3.3.2 Serum ferritin

Serum ferritin, as an indicator of body iron stores, was previously measured in 18,432 participants using blood stored at baseline. Levels were estimated using an AutoDELFIA ferritin kit (Wallace Oy, Turku, Finland) for the two-step time-resolved fluoroimmunoassay [(218)]. The coefficient of variation between batches was 5.8% at 4.6 µg/l and 6.7% at 355 µg/l.

2.3.4 Heel ultrasound measurements

In EPIC-Norfolk, estimates of heel ultrasound had previously been undertaken in subjects attending the second health check in 1997-2000. Measurements were performed with a CUBA Clinical Ultrasonometer (McCue Ultrasonics, Winchester, UK) **(Figure 2.4)** at least twice on each heel and the mean value of the left and right measure were calculated for the ultrasound parameters BUA (in dB/MHz) and VOS (in m/s). Ultrasound was chosen over DXA based on lower cost and the portability of the equipment in the large sample size of EPIC-Norfolk. BUA measures bone density as well as its structural organisation; whereas VOS is a measure of both bone density and bone stiffness [(62, 63)]. Higher values for both heel ultrasound parameters are an indication of greater bone quality.

Figure 2.4: The measurement of BUA and VOS at the heel bone using ultrasound.



CUBA Clinical Ultrasonometer, McCue Ultrasonics, Winchester, UK.

2.3.5 DXA measurements

BMD measurements of the total hip region using DXA had previously been performed in a small sub-sample of the full EPIC-Norfolk cohort (*n*=1511) on the same day as the heel ultrasound measurements [(68)], and those data were used in the sub-study of combining vitamin C intake and status measurements (Chapter 8, page 212). DXA determines the average amount of bone mineral of the scanned area in a two dimensional format [(60)], and it is considered the gold standard measurement of bone density for the diagnosis of osteoporosis [(63)]. All hip BMD measurements (in g/cm^2) were completed by the same operator using a Hologic 1000 W bone densitometer (Hologic, Bedford, MA, USA), and all scans were reviewed by an independent operator to ensure consistency [(219)].

2.3.6 Fractures

EPIC-Norfolk is linked to the East Norfolk health authority database (ENCORE) [(68)] which records all hospital contacts throughout England and Wales via the unique NHS number. Using ENCORE, diagnostic codes had previously been used to identify osteoporotic fractures by site which had been occurring in the cohort in March 1997-2009. For the present prospective study, data for fractures at the hip, spine and wrist were available for analysis. The combined total number of fractures at these three sites was also derived and will hereafter be referred to as total fractures.

2.4 Statistical Analyses

2.4.1 Covariates

As discussed in Chapter 1 (pages 26-28), there are numerous factors which may have detrimental effects on bone health. Depending on both the evidence in the literature and the availability of data in the EPIC-Norfolk cohort, a number of risk factors were identified to potentially impact on bone health, and those were chosen as covariates in the following cross-sectional and prospective diet-bone investigations. The following covariate models were developed:

- **Unadjusted.**
- **Model 1 - Biological and lifestyle factors:** age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status (women), HRT (women).
- **Model 2 - Dietary factors:** Model 1 + total energy intake, dietary calcium intake, use of calcium and/or vitamin D supplements.

At first, all analyses were performed unadjusted in order to obtain the crude relationships between exposure and outcome measures. Then, a model containing important biological and lifestyle covariates was applied to the sex-specific analyses (Model 1), before dietary exposures were added to the model (Model 2).

Age, BMI and the intake of energy and calcium from foods were considered a continuous variable. Binary variables with "Yes" and "No" options were family history of osteoporosis, steroid medication and the use of calcium and vitamin D supplements. Smoking status was defined as "current", "former" and "never smoking" and physical activity was defined as "active", "moderately active", "moderately inactive", and "inactive". Women were classed into categories of menopausal status as "pre-menopausal", "early peri-menopausal" (less than one year), "late peri-menopausal" (1-5 years) and "postmenopausal" (more than five years). HRT use in women was defined as "current", "former" and "never" categories. Several confounding factors, including previous fragility fractures and a family history of fracture, were not available from the current dataset and hence could not be included in the present prospective investigations. Race was not considered to be a confounder as the EPIC-Norfolk cohort comprises almost exclusively Caucasian men and women.

2.4.2 Investigating associations between diet and heel ultrasound

To explore potential cross-sectional associations between nutrient intakes and nutrient status in blood with heel ultrasound, Pearson correlation coefficients were calculated. The dependent

variables BUA and VOS were plotted against quintiles of each independent nutrient variable of interest. For this, mean (±SE) BUA and VOS were stratified by sex-specific quintiles of nutrient intake and status using multiple regression and were adjusted for confounding factors, as discussed above. Differences in adjusted mean BUA and VOS between extreme quintiles of nutrients referent to the lowest quintile were determined unless stated otherwise. A test for a linear trend gave an indication of the strength of the associations. The use of specific quantiles (tertiles, quartiles or quintiles) depended on the distribution of the data. Quintiles were chosen where possible because it gave a finer discrimination of the nutrient intakes. All statistical analyses were stratified by sex as significant sex differences in bone health have previously been established [220]. In order to be able to put the present findings in to context, a sample size calculation was performed post-hoc for the study of vitamin K_1 intake and heel ultrasound (Chapter 4). These analyses were chosen because the heel ultrasound study was based on a smaller dataset than the fracture study and the additional exclusion of participants with missing aspirin data in this chapter resulted in the smallest dataset compared to the other nutrient chapters.

2.4.3 Investigating associations between diet and fracture risk

To investigate potential associations between nutrient intakes and nutrient status in blood with fracture risk, mean nutrient intake and status were calculated for fracture and non-fracture participants stratified by sex and fracture site. An unpaired *t*-test was used to test for differences in nutrient intake and status between the two groups. Numbers and percentages of fractures (hip, spine, wrist and the combined total) were tabulated by quintiles of each nutrient. Then, Cox proportional hazard ratios of fracture risk with 95% confidence intervals were calculated for unadjusted and adjusted means, as discussed above. The Cox model used a Prentice-weighted approach as this allowed to account for the case-cohort design of the present prospective study [221]. Differences between extreme quintiles of nutrient intake and status were determined unless stated otherwise. The linearity of the associations was indicated using *P* for trends. All statistical analyses were stratified by sex as previously discussed.

2.4.4 Investigating associations in the sub-study

The sub-study combined intake and biomarker measurements of vitamin C to investigate the association with heel ultrasound and fracture risk (Chapter 8). For this, standardised measures of BUA, VOS and DXA were calculated in order to compare the regression coefficients by dividing the bone measures by their standard deviation. Participants were ranked according to their vitamin C intake and status measures, before those were combined [222]. Due to a smaller sample

size, quartiles of vitamin C were determined, and those fitted the data better than tertile and quintile groups. Adjusted linear regression analyses were performed for quartiles of vitamin C intake from the 7dDD and the FFQ, plasma vitamin C concentrations and their respective combinations with the standardised bone measures. Moreover, adjusted Prentice-weighted Cox proportional hazards of total fracture risk were calculated for quartiles of vitamin C intake from the 7dDD and the FFQ, plasma vitamin C concentrations and their respective combinations. The adjusted regression coefficients and hazard ratios were compared for the linear trend across all quartiles and for differences between the higher quartiles with the lowest quartile. All statistical analyses were stratified by sex as previously discussed, with the exception of the DXA analyses in Chapter 8 (pages 222-223) which were undertaken in the combined sample of men and women but adjusted for sex due to the small sample size.

All statistical analyses were performed using STATA (Statistical Software: Release 11; 2009, StataCorp LP). A *P* value of <0.05 was considered statistically significant in all analyses.

CHAPTER 3

THE DERIVATION OF THE DATASET, BASIC DESCRIPTIVES OF THE COHORT AND THE RELATIONSHIP BETWEEN THE COVARIATES AND FRUIT AND VEGETABLE INTAKES WITH BONE HEALTH IN THE EPIC-NORFOLK STUDY

3.1 Overview of the chapter rationale and methodology

This chapter includes information on four different aspects of the EPIC-Norfolk sub-cohort datasets. It aims to i) provide information on the derivation of the two datasets, ii) determine how representative the EPIC-Norfolk sub-cohort is of the general UK population and how it compares to other studies, iii) investigate how the covariates chosen in Chapter 2 are related to bone health in this cohort, and iv) explore the associations between dietary intakes of fruit and vegetables with bone health for comparison reasons with the following chapters.

First, the derivation of two EPIC-Norfolk sub-cohort datasets is described. As discussed in Chapter 2, two different datasets were created to accommodate the two different types of studies: i) the cross-sectional investigation of heel ultrasound undertaken in a random sub-cohort of 4000 subjects, and ii) the prospective investigations of fracture risk undertaken in a case-cohort of 5319 participants. The present chapter describes the derivation of the final number of men and women in each dataset, which were subsequently used for the analyses in the following chapters, and highlights the number of missing values at each stage of the derivation process.

Next, the baseline characteristics, including dietary variables, of the EPIC-Norfolk case-cohort study were assessed. All analyses were stratified by sex in order to ensure they were consistent with the sex-specific bone health analyses. As variables were normally distributed, the mean and standard deviation was calculated for continuous variables of the first health check including age, BMI, heel ultrasound (second health check), concentrations of plasma vitamin C and serum ferritin, energy intake, as well as intakes of the macronutrients and a selection of micronutrients. Differences in these characteristics between men and women were determined using an unpaired *t*-test. For binary and categorical variables, the frequencies and proportions were determined for first health check data including the number of completed days of the 7dDD, smoking, physical activity, menopausal status and HRT use in women, a family history of osteoporosis, the use of steroid medication, calcium and vitamin D supplements and the occurrence of fractures during follow-up. Chi square tests were used to determine differences in these binary and categorical variables between men and women, except for menopausal status and HRT use in women which was assessed for differences between the different groups of each variable. Similar to determining baseline differences between men and women, unpaired *t*-tests and chi square tests were also used to investigate differences in descriptive variables between participants who had experienced a fracture during the follow-up period and those who remained free from fractures. Moreover, as the number of men and women in the fractured and non-fractured groups were uneven, baseline differences between the two groups were also determined using regression analyses adjusted for sex.

Following the determination of baseline characteristics, the EPIC-Norfolk sub-cohort was compared to the general UK population as well as other populations as reported in the literature. For this, predominantly data from the National Diet and Nutrition Survey (NDNS) published in 1998 were used for comparing dietary intakes as well as nutrient status measurements [223]. Despite more recent national data being available, the comparative data from 1998 was chosen due to the initial recruitment time of EPIC-Norfolk participants in 1993-1997. However, the data were also evaluated in relation to the latest NDNS survey to assess comparability to current UK intakes [224]. Nutrient intakes were also compared to national recommendations using Dietary Reference Values [225].

Next, this chapter aimed to explore the relationship between risk factors for osteoporosis and bone health in this population. As discussed in Chapter 1 (pages 26-28), previous studies have highlighted many risk factors for the development of osteoporosis and fractures [27, 30, 31, 79-82, 84-86, 88, 97-99, 126]. Based on the evidence in the literature and the availability of data in the EPIC-Norfolk cohort study, a number of important risk factors were decided on to include as covariates in the present investigations of diet and bone health; and these included: age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. In this chapter, the relationship between these covariates with both heel ultrasound measurements and with fractures was explored in the present EPIC-Norfolk sub-cohort. For this, regression analyses were used to determine associations between the covariates and heel ultrasound measurements, where the *P*-trend was indicative of the linearity of the relationship. Moreover, associations between the covariates and total fractures were investigated using chi square tests. All investigations were conducted for men and women separately as previous studies have reported bone-specific sex differences [27].

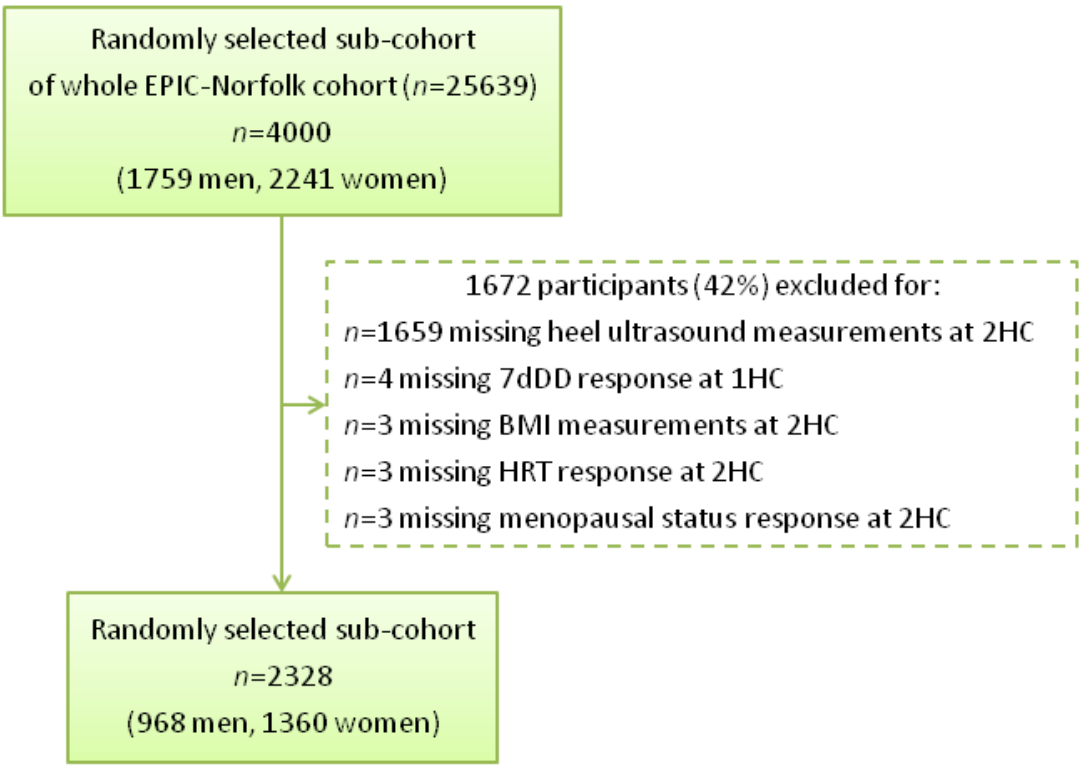
Finally, this chapter also investigated potential associations between dietary intakes of fruit and vegetables with bone health for comparison reasons with the following chapters, which will explore nutrient-bone relationships of predominantly plant-based nutrients. Linear regression analyses were used to determine the cross-sectional association between fruit intake, vegetable intake and their combined intake (F&V) with heel ultrasound measurements; whereas Prentice-weighted Cox proportional hazards of fracture risk (hip, spine, wrist and total) were used in the prospective study. All analyses were adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements.

3.2 Derivation of the dataset

3.2.1 Cross-sectional heel ultrasound study

The cross-sectional study of heel ultrasound aimed to determine potential associations between dietary intakes and nutrient status with measures of heel ultrasound. It was based on a random sub-cohort of 4000 participants which was representative of the 25,639 participants who had attended the initial health check in 1993-1997. The smaller representative dataset was used for all analyses to ensure that no selection bias was introduced into the present study because data entry as well as data cleaning and processing were not yet complete at the time of writing. As shown in **Figure 3.1**, those with missing 7dDD data from the first health check and with missing heel ultrasound and covariate information from the second health check (1997-2000) were identified. Participants who did not attend the second health check, and thus had missing heel ultrasound measurements, were excluded (*n*=1659). Those with missing responses for smoking status at the second health check were recoded to the current smoking category (*n*=11), as these participants were likely smokers who only smoked occasionally and were unsure about answering this question in the lifestyle questionnaire. Women with missing menopausal status responses were recoded to the post-menopausal category if they were at least 55 years of age, as most women have reached menopause at this age, or if they were current or former users of HRT at the second health check (*n*=34). A further seven women, who were younger than 50 years and had never used HRT, were recoded to the pre-menopausal category. Four participants with missing baseline 7dDD and participants with missing second health check data for BMI (*n*=3), HRT use (*n*=3) and menopausal status (*n*=3) were also excluded from the analyses. Hence, a total of 2327 participants (968 men and 1360 women) remained for the present cross-sectional investigations of nutrient intakes and heel ultrasound.

Figure 3.1: Flow chart showing the number of participants of the randomly selected EPIC-Norfolk sub-cohort included in the cross-sectional study to determine potential diet and heel ultrasound associations.

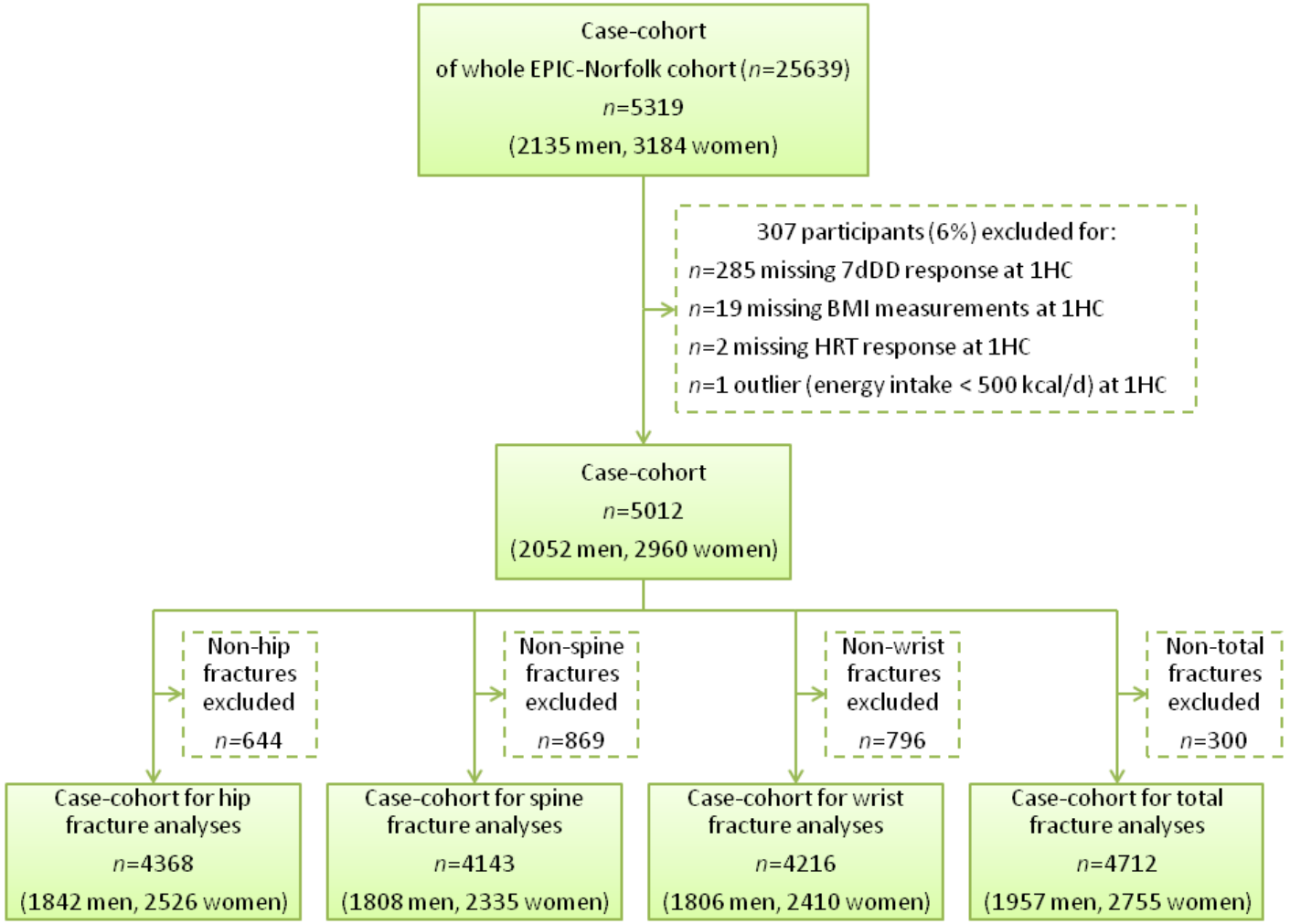


Abbreviations: 1HC, first health check (1993-1997); 2HC second health check (1997-2000).

3.2.2 Prospective fracture risk investigations

Figure 3.2 illustrates the number of participants of the EPIC-Norfolk cohort included in the present prospective case-cohort fracture risk investigations. The case-cohort rather than the full EPIC-Norfolk cohort was used in these investigations due to the limited availability of the data. The initial case-cohort sub-sample contained data for a total of 5319 participants (2135 men and 3184 women), representing 21% of the full cohort. The dataset was searched for missing 1HC covariate data and recoding of these was undertaken where appropriate. Participants with missing information for smoking were recoded to the current smoking category (*n*=52), for the same reasons mentioned above. Women with unknown menopausal status were recoded to post-menopausal if they were at least 55 years of age or were current or former users of HRT (*n*=3). Next, subjects were excluded from this study if they had missing 1HC data for one or more of the following variables: 7dDD (*n*=285; 5.4%), BMI (*n*=19; 0.4%) and HRT in women (*n*=2; <0.01%). The dataset was also screened for potential outliers and one participant with a very low energy intake (322 kcal/d) was removed from the dataset, leaving a case-cohort sample of 5012 participants (2052 men and 2960 women). For the fracture-site-specific analyses, participants were excluded from the study if they had sustained a fracture at a different site and were not part of the random sub-cohort. Hence, investigations of hip fractures were conducted in 4368 participants, spine fractures in 4143 participants, wrist fractures in 4216 participants and the combined total fractures of these three sites in 4712 participants.

Figure 3.2: Flow chart showing the number of EPIC-Norfolk participants included in the case-cohort study of fracture risk.



Abbreviations: 1HC, first health check (1993-1997). Total fractures are the number of fractures at the hip, spine and wrist combined. The numbers excluded for the case-cohort differ between the different fracture sites as participants were excluded from the study if they had suffered a fracture at a different site and were not part of the random sub-cohort.

3.3 Baseline characteristics and representativeness of the EPIC-Norfolk case-cohort with other populations

In the full EPIC-Norfolk cohort of 25,639 participants who had attended the first health check, a total of 1083 fractures (294 in men, 789 in women) had been recorded at the hip, spine and wrist between March 1997 and March 2009. This gave a cumulative incidence of 4.2% over the median follow-up of 12.6 years. Following the exclusion of participants with missing data, the present EPIC-Norfolk case-cohort sample included 5012 participants, 60% of which were women. Their baseline characteristics are shown in **Table 3.1**. The mean±SD age was 60±10 years in both men and women at baseline. BMI differed significantly between men and women (26.5±3.3 kg/m^2 *vs.* 26.2±4.3 kg/m^2, *P*=0.005), and the mean BMI values were comparable with those of the national population at the time of recruitment [(226)]. Less than 1% of the population were underweight (BMI<18.5 kg/m^2), whereas 60% were either overweight or obese (BMI ≥ 25 kg/m^2). In those men and women with heel ultrasound measurements, there was a strong correlation of 0.74 between BUA and VOS (*P*<0.05). Mean heel ultrasound measurements were significantly lower in women (BUA: 70±17 dB/MHz; VOS: 1620±41 m/s) than in men (BUA: 89±18 dB/MHz; VOS: 1642±41 m/s; *P*<0.001). Women were more likely to experience a total fracture than men (21% *vs.* 12%, *P*<0.001). Fifteen percent and 65% of women were pre-menopausal and post-menopausal, respectively. Seventeen percent of women were current users of HRT, although most (71%) had never previously received HRT. Only 12% of participants were current smokers, and 33% of men and 56% of women indicated that they had never smoked. Early work on the EPIC-Norfolk cohort [(209)] reported that this study population had a much lower proportion of current smokers across different age groups compared to the general population of England at the time of recruitment (men: 10-15% *vs.* 20-28%; women: 8-14% *vs.* 18-27%) [(226)].

Table 3.1: Baseline characteristics of the EPIC-Norfolk case-cohort sample.

	Men ***n=2052***		**Women** ***n=2960***		
	Mean	**(SD)**	**Mean**	**(SD)**	***P***
Age (yrs)	60	(10)	60	(10)	*P*=0.52
BMI (kg/m^2)	26.5	(3.3)	26.2	(4.3)	*P*=0.005
BUA (dB/MHz) *	89	(18)	70	(17)	*P*<0.001
VOS (m/s) *	1642	(41)	1620	(41)	*P*<0.001
Plasma vitamin C (µmol/l)†	46.4	(18.1)	58.1	(20.0)	*P*<0.001
Serum ferritin (ng/ml)‡	115.6	(85.2)	64.7	(52.8)	*P*<0.001
	(n)	**%**	**(n)**	**%**	
Prevalence of fractures					
Total (hip, spine and wrist)	(248)	12.1	(616)	20.8	*P*<0.001
Hip	(112)	5.5	(339)	11.5	*P*<0.001
Spine	(78)	3.8	(124)	4.2	*P*=0.49
Wrist	(70)	3.4	(218)	7.4	*P*<0.001
Smoking history					*P*<0.001
Current smoker	(248)	12.1	(369)	12.5	
Former smoker	(1137)	55.4	(948)	32.0	
Never smoked	(667)	32.5	(1643)	55.5	
Physical activity					*P*<0.001
Inactive	(640)	31.2	(981)	33.1	
Moderately inactive	(500)	24.4	(941)	31.8	
Moderately active	(454)	22.1	(613)	20.7	
Active	(458)	22.3	(425)	14.4	
Menopausal status					*P=N/A*
Pre-menopausal	-	-	(433)	14.6	
Peri-menopausal (<1 yr)	-	-	(140)	4.7	
Peri-menopausal (1-5 yrs)	-	-	(473)	16.0	
Post-menopausal	-	-	(1914)	64.7	
Hormone replacement therapy					*P=N/A*
Current user	-	-	(508)	17.2	
Former user	-	-	(347)	11.7	
Never used	-	-	(2105)	71.1	
Family history of osteoporosis	(60)	2.9	(165)	5.6	*P*<0.001
Use of steroids	(73)	3.6	(129)	4.4	*P*=0.16
Use of calcium supplements	(26)	1.3	(168)	5.7	*P*<0.001
Use of vitamin D supplements	(445)	21.7	(930)	31.4	*P*<0.001
Completed days of 7dDD					*P*=0.003
1-2	(176)	8.6	(180)	6.1	
3-6	(31)	1.5	(62)	2.1	
7	(1845)	89.9	(2718)	91.8	

Abbreviations: N/A, not applicable.Values are means (standard deviations) or frequencies. P-values were determined using unpaired t-tests for continuous variables and chi square tests for binary and categorical variables.

** n=1130 men and n=1752 women.*

† n=1842 men and n=2554 women.

‡ n=1450 men and n=1963 women.

Women were less physically active than men, with 44% of men, but only 35% of women, being active or moderately active (*P*<0.001). More women (5.6%) than men (2.9%) reported a family history of osteoporosis (*P*<0.001) and 4% of the population reported current use of steroid medication.

Most participants (91%) completed the full seven days of the diet diary, and a preliminary analysis showed that there were no differences in dietary intake compared to diaries completed for fewer days. Data from the 7dDDs showed that more women than men used calcium supplements (5.7% *vs.* 1.3%; *P*<0.001) and vitamin D supplements (31.4% *vs.* 21.7%; *P*<0.001). Measurements of nutrient status in blood were available for a smaller number of participants than the dietary intakes in the EPIC-Norfolk case-cohort. In this population, men had significantly higher serum ferritin levels than women (116±85 *vs.* 65±53 ng/ml; *P*<0.001; *n*=3413); and compared to the general UK population of free-living individuals older than 65 years at the time of recruitment (men: 122±126 ng/ml; women: 80±79 ng/ml), mean serum ferritin concentrations were lower in the present study population [(223)]. Mean plasma vitamin C concentrations (*n*=4396) in the EPIC-Norfolk sub-cohort were significantly lower in men than in women (46±18 *vs.* 58±20 µmol/l; *P*<0.001), and this is in agreement with previous studies [(227)]. Mean plasma vitamin C concentrations were higher in this population compared to the national population average (men: 39±22 µmol/l; women: 49±26 µmol/l) [(223)].

The dietary intakes of the EPIC-Norfolk case-cohort study population are shown in **Table 3.2**. Total energy intake was significantly higher for men (2244±514 kcal/d) than for women (1679±389 kcal/d; *P*<0.001). Mean energy intakes were within range of the estimated average requirement (EAR) for the UK population aged 19 years and over for men (2100-2550 kcal/d) and slightly below the EAR for women (1810-1940 kcal/d) [(225)]. Intakes of carbohydrate, protein and fat, respectively, provided 49%, 15% and 34% of total energy intake in men and 50%, 15% and 34% in women. This was comparable to the national guideline of 47%, 15% and 33%, respectively [(225)]. In the EPIC-Norfolk sub-cohort, the mean daily combined fruit and vegetable consumption was 250±164 g in men and 284±169 g in women, which is equivalent to around three servings per day. However, less than one fifth (17%) of the study population followed the current international guideline of eating at least five portions (400g) of fruit and vegetables per day [(228)]. When fruit and vegetable intakes were investigated separately, it was found that women consumed significantly more fruit (175±135 *vs.* 143±132 mg/d; *P*<0.001) but not vegetables (110±71 *vs.* 107±74 mg/d; *P*=0.20) than men.

Table 3.2: Dietary intakes of the EPIC-Norfolk case-cohort sample at baseline.

	Men ***n=2052***		**Women** ***n=2960***		
	Mean	**(SD)**	**Mean**	**(SD)**	***P***
Fruit and vegetables (g/d)	250	(164)	284	(169)	*P*<0.001
Fruit (g/d)	143	(132)	175	(135)	*P*<0.001
Vegetables (g/d)	107	(74)	110	(71)	*P*=0.20
Energy (kcal/d)	2244	(514)	1679	(389)	*P*<0.001
Carbohydrates (g/d)	273	(73)	211	(54)	*P*<0.001
Protein (g/d)	82	(18)	65	(14)	*P*<0.001
Fat (g/d)	86	(26)	64	(21)	*P*<0.001
Alcohol (g/d)	17	(22)	8	(12)	*P*<0.001
Fibre (g/d)	16	(6)	14	(5)	*P*<0.001
Calcium (mg/d)	915	(296)	760	(253)	*P*<0.001
Magnesium (mg/d)	322	(93)	265	(73)	*P*<0.001
Iron (mg/d)	13	(4)	11	(3)	*P*<0.001
Potassium (mg/d)	3449	(821)	2964	(689)	*P*<0.001
Vitamin C (mg/d)	86	(52)	89	(51)	*P*=0.020
Vitamin K_1 (µg/d)	95	(59)	87	(52)	*P*<0.001

P-values were determined using unpaired t-tests.

In line with men having higher energy intakes than women, dietary intakes for all macro- and micronutrients were also greater in men than women (*P*<0.001), except for dietary vitamin C **(Table 3.2)**. Mean vitamin C intake was significantly higher in women than in men (89±51 *vs.* 86±52 mg/d; *P*<0.020), and this was in line with women having significantly higher plasma vitamin C levels (58±20 *vs.* 46±18 µmol/l; *P*<0.001). Most participants (87%) had intakes greater than the UK Reference Nutrient Intake (RNI) of 40 mg/d and only a very small percentage of men and women (0.4%) had vitamin C intakes below the lower RNI (LRNI) of 10 mg/d [(225)]. However, mean intakes were lower in this cohort than in most previous studies in UK, US and Japanese populations using FFQs to assess intake [(91, 133, 229, 230)]. Similarly, mean dietary vitamin K_1 intakes were consistently lower in the EPIC-Norfolk cohort compared to previous publications of US, UK and other European populations [(154-156)]. Moreover, intake ranges were lower in the EPIC-Norfolk men than in US men [(155)], although intake ranges in women were comparable with previous studies in UK and US women [(154, 155)]. In the EPIC-Norfolk sub-cohort, 91% of men had adequate dietary iron intakes (≥8.7 mg/d) and only three men had iron intakes below the LRNI of 4.7 mg/d [(225)]. In contrast to men, dietary iron intake recommendations of 8.7 mg/d for women aged 50 years and older and 14.8 mg/d for menstruating women up to the age of 50 years were only met by 51% and 13%, respectively. Only very few older women (1%) had dietary iron intakes below the LRNI guideline of 4.7 mg/d, whereas 14% of younger women did not meet the LRNI of 8 mg/d. Higher dietary iron intakes in men compared to women were also reflected by men having significantly higher serum ferritin levels than women (116±85 *vs.* 65±53 ng/ml; *P*<0.001). In comparison to the general UK population at the time of recruitment, mean dietary iron intakes

were slightly higher in participants of the EPIC-Norfolk cohort (men: 11 *vs.* 13 mg/d; women: 9 *vs.* 11 mg/d) [223].

These results are still relevant to current dietary intakes as the data are still comparable to more recent data for people older than 65 years from the National Diet and Nutrition Survey (NDNS) Rolling Programme (2008/2009 – 2009/2010) [224]. For example, the dietary iron intake of the general UK population was similar between the NDNS survey in 1994-1995 [223] and 2008-2010 [224] (men: 11.0 *vs.* 11.3 mg/d; women: 8.6 *vs.* 9.5 mg/d), and intakes remained slightly lower than that of the EPIC-Norfolk participants (men: 13 mg/d; women: 11 mg/d).

3.4 Characteristics of participants with and without fractures

Table 3.3 shows a comparison of those baseline demographics between fracture and non-fracture subjects which were used as covariates in further analyses. Participants with a fracture at the hip, spine or wrist over a median follow-up of 12.6 years were significantly older than non-fracture subjects (65±8 *vs.* 59±9 years, $P<0.001$). Fracture subjects had significantly lower BUA (68±19 *vs.* 80±19 dB/MHz) and VOS (1607±42 *vs.* 1634±41 m/s) compared to participants who remained free from fractures (all $P<0.001$). Although BMI did not differ between the two groups, total energy intake was significantly lower in fracture subjects (1799±521 kcal/d) than in those without fractures (1940±517 kcal/d; $P<0.001$). Fracture subjects were also more likely to be inactive and report the use steroid medication ($P\leq0.001$). As the number of fractured participants varied by sex, the comparison of baseline demographics between those who had a fracture and those who did not was also undertaken with the adjustment for sex in the chi square tests. The results were comparable to the non-adjusted analyses, except for total energy intake. The unadjusted mean difference in energy intake between the two groups (141 kcal/d), with non-fracture subjects having significantly higher intake ($P<0.001$), were smaller when sex differences were accounted for (54 kcal/d).

Table 3.3: Characteristics of participants with and without a total fracture [1].

	Non-fracture subjects (*n*=3848) *(1709 men, 2139 women)*				**Fracture subjects (*n*=864) *(248 men, 2139 women)***					
	Mean	**(SD)**	**Sex-adjusted Mean**	**(SD)**	**Mean**	**(SD)**	**Sex-adjusted Mean**	**(SD)**	***P***	***Sex-adjusted P***
Age (yrs)	59	(9)	59	(9)	65	(8)	65	(9)	*P*<0.001	*P*<0.001
BMI (kg/m^2)	26.3	(3.9)	26.3	(3.9)	26.1	(4.2)	26.2	(4.0)	*P*=0.17	*P=0.31*
BUA (dB/MHz) †	80	(19)	80	(22)	68	(19)	70	(23)	*P*<0.001	*P*<0.001
VOS (m/s) †	1634	(41)	1634	(52)	1607	(42)	1609	(55)	*P*<0.001	*P*<0.001
Total energy intake (kcal/d)	1940	(517)	1924	(442)	1799	(521)	1870	(445)	*P*<0.001	*P*=0.001
	%	**(n)**			**%**	**(n)**				
Smoking history									*P*=0.47	
Current smoker	12.4	(476)			12.1	(105)				
Former smoker	42.2	(1624)			40.2	(347)				
Never smoked	45.4	(1748)			47.7	(412)				
Physical activity									*P*<0.001	
Inactive	30.0	(1155)			42.5	(367)				
Moderately inactive	28.8	(1108)			27.8	(240)				
Moderately active	22.6	(869)			16.7	(144)				
Active	18.6	(716)			13.1	(113)				
Family history of osteoporosis	4.5	(172)			4.6	(40)			*P*=0.84	
Use of steroids	3.4	(131)			5.9	(51)			*P*=0.001	
Use of calcium supplements	3.9	(150)			3.5	(30)			*P*=0.56	
Use of vitamin D supplements	27.2	(1047)			29.9	(258)			*P*=0.12	

Values are means (standard deviations) or frequencies. P-values were determined using unpaired t-tests for continuous variables and chi square test for binary and categorical variables.

[1] The sample size of 4712 is the number of participants included in the analysis of total fractures.

† Data for a smaller number of participants were available for analysis: 453 fracture subjects and 2256 non-fracture subjects.

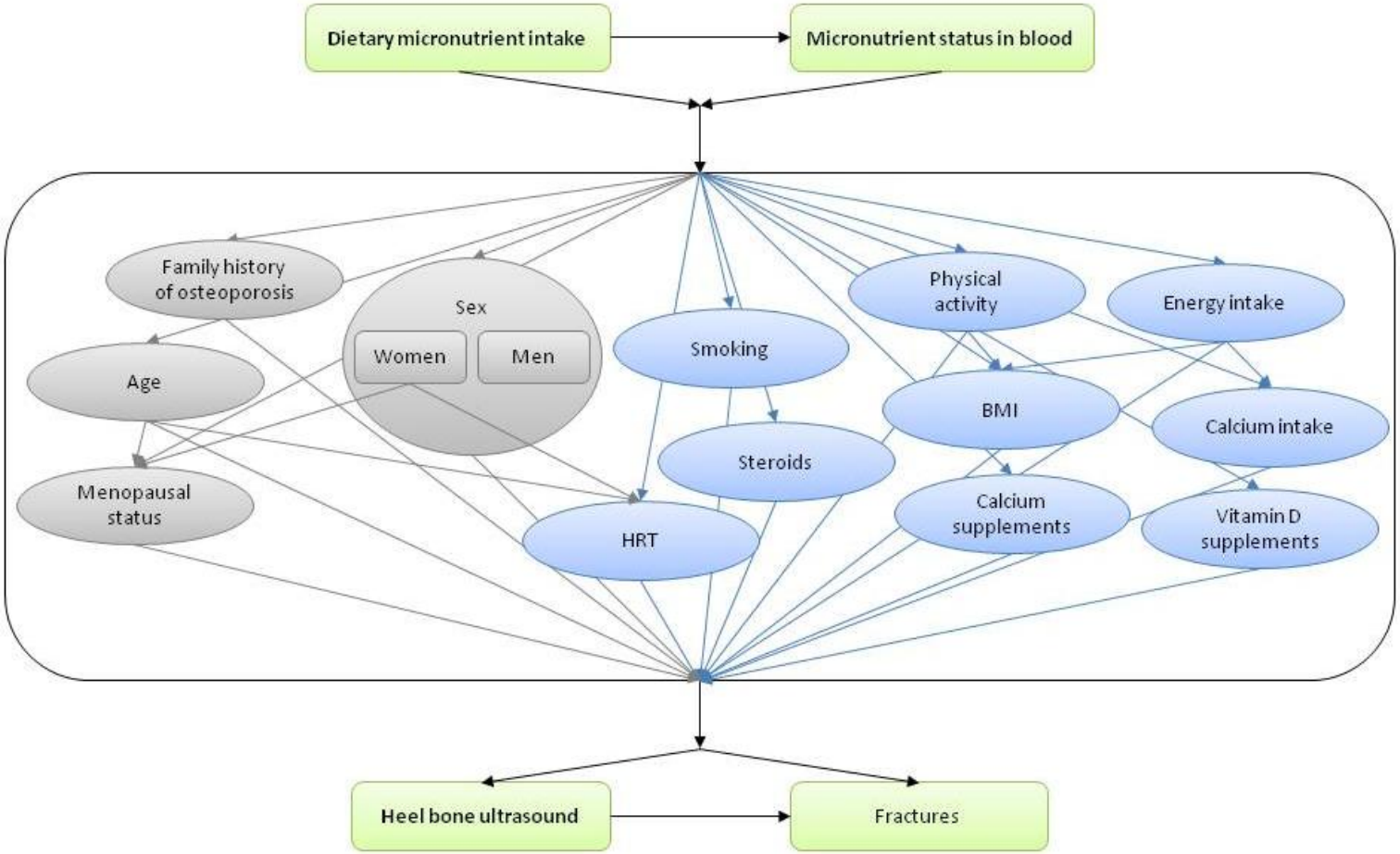
3.5 The covariates and their relation to bone health

As previously discussed (Chapter 1, pages 26-28), a number of important risk factors for bone health were identified and those were included as covariates in the present investigations of diet and bone health **(Figure 3.3)**.

3.5.1 Heel ultrasound

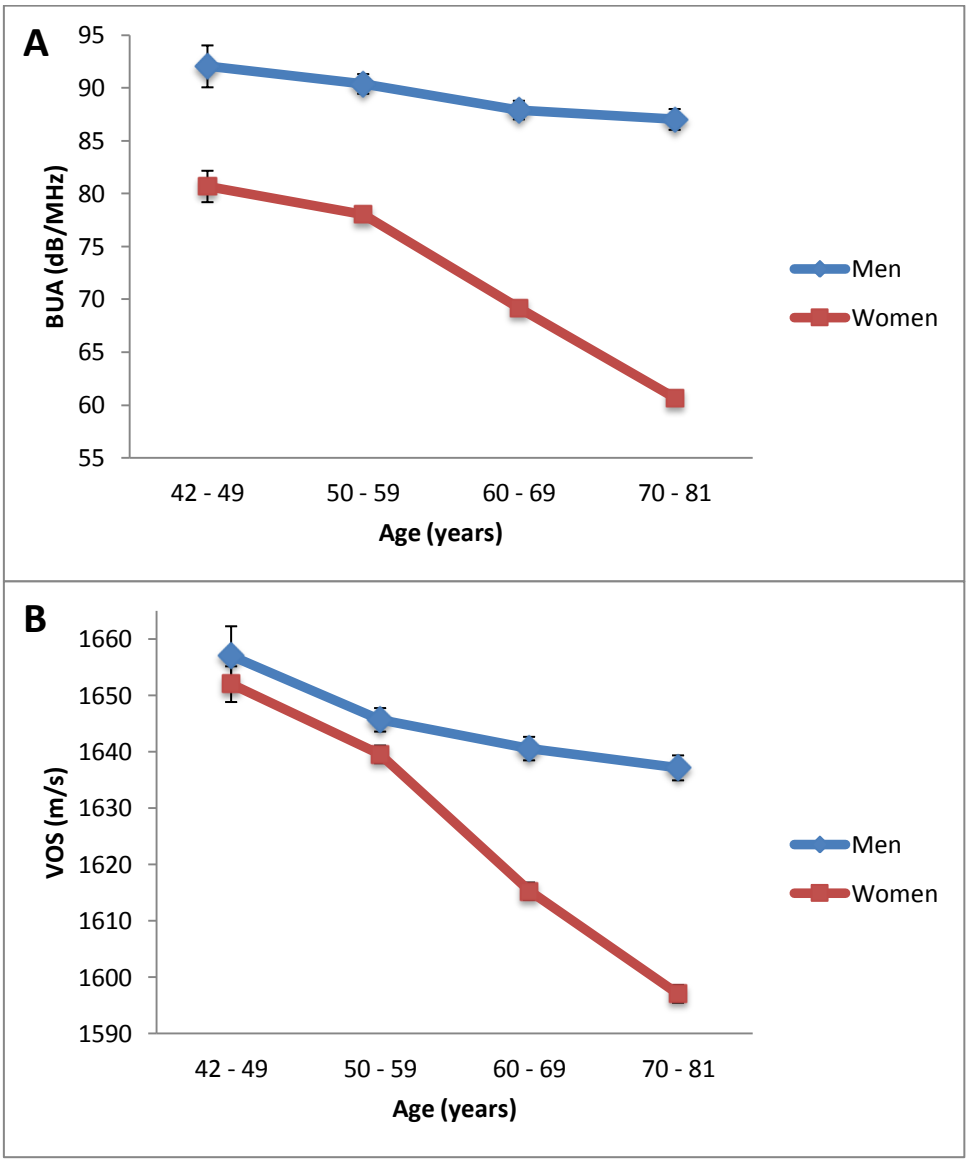
Age was assessed as a categorical variable in 10-year intervals (<50 years, ≥50 to <60 years, ≥60 to <70 years, and ≥70 years), as a 10-year age increase and decrease in BMD has been associated with the risk of developing a fracture [(110)]. In the randomly selected EPIC-Norfolk sub-cohort, an increase in age by approximately 10 years was significantly associated with lower BUA (β±SE: β -1.7±0.6 dB/MHz, *P*=0.002) and VOS (β -5.4±1.3 m/s, *P*<0.001) in men, and more with lower BUA (β -7.9±0.4 dB/MHz, *P*<0.001) and VOS (β -20.2±0.9, *P*<0.001) in women **(Figure 3.4)**. Men with a family history of osteoporosis compared to those without had significantly lower BUA (83±2.9 *vs.* 89±0.5± dB/MHz, *P*=0.040) and VOS (1622±6.7 *vs.* 1643±1.2 m/s, *P*=0.002) after age adjustment. A family history of osteoporosis was not associated with lower heel ultrasound in women. Categories of BMI were defined as: normal weight (<25 kg/m^2), overweight (25 – 29.9 kg/m^2) and obese (>30 kg/m^2). Increasing BMI was significantly associated with higher age-adjusted BUA in women (β 5.7±0.5 dB/MHz, *P*<0.001), and the associations were almost significant in men (β 1.4±0.8 dB/MHz, *P*=0.068). VOS was also positively associated with higher BMI in women (β 4.4±1.2 m/s, *P*<0.001), but unexpected significant associations between higher BMI and decreasing VOS were observed in men (β -8.5±1.8 m/s, *P*<0.001). Current female smokers had significantly lower mean BUA (67.2±1.2 dB/MHz) compared to former smokers (70.8±0.6 dB/MHz, *P*=0.007) and never smokers (70.4±0.5 dB/MHz, *P*=0.011), after adjustment for age. Similarly, current male smokers had almost significantly lower VOS compared to never smokers (1636±4.2 *vs.* 1645±2.0 m/s, *P*=0.054). Higher levels of physical activity were significantly associated with higher age-adjusted BUA (β 1.2±0.5 dB/MHz, *P*=0.012) and VOS (β 4.6±1.1 m/s, *P*<0.001) in men, but not in women. Both menopausal status and HRT were significantly associated with heel ultrasound in women. Measurements of VOS decreased by 4.6 m/s across categories of menopausal status (defined as pre-menopausal, early peri-menopausal, late peri-menopausal and postmenopausal) after adjustment for age and HRT use (*P*=0.003). Moreover, current users of HRT had significantly higher BUA and VOS compared to former users (BUA β -4.0±1.2 dB/MHz, *P*=0.001; VOS β -11.9±2.9 m/s, *P*<0.001) and never users (BUA β -6.9±1.0 dB/MHz, *P*<0.001; VOS β -16.3±2.3 m/s, *P*<0.001) of HRT. The use of steroid medication, calcium supplements as well as vitamin D supplements was not associated with age-adjusted heel ultrasound in either sex. Moreover, dietary calcium intake was not found to be associated with BUA and VOS in this population, after adjustment for age and energy intake.

Figure 3.3: Confounding factors in the relationship between diet and bone health.



Confounders were colour-coded according to their modifiability: non-modifiable biological factors are shown in grey and modifiable lifestyle factors appear in blue.

Figure 3.4: The effects of age on mean BUA **(A)** and VOS **(B)** in the EPIC-Norfolk case-cohort sample.

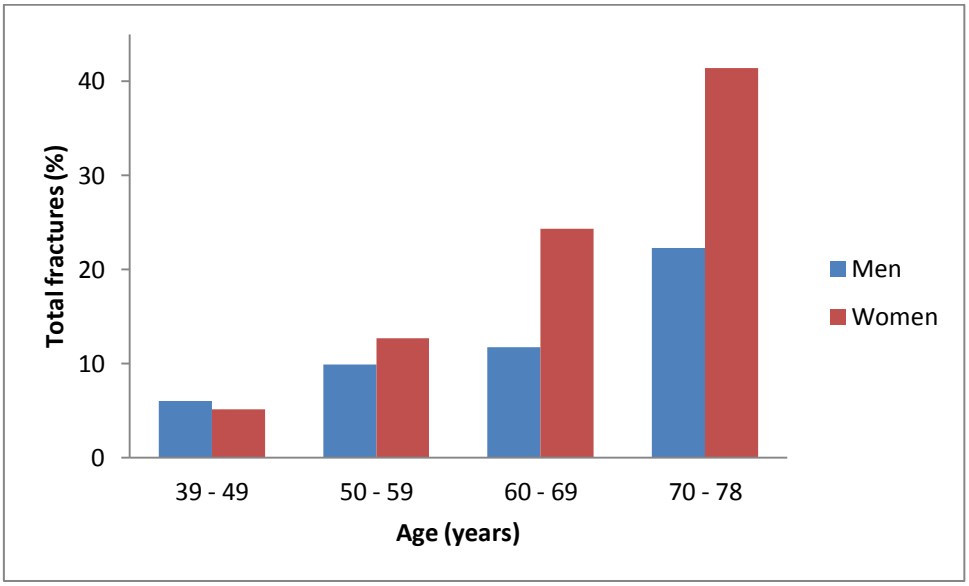


Sex-specific associations between increasing age and heel ultrasound were determined from regression analyses. The relationship was linear in both men (BUA P-trend=0.002; VOS P-trend<0.001) and women (all P-trend < 0.001). n=2328.

3.5.2 Fractures

In order to determine the effects of increasing age on the risk of fractures in this cohort, age was divided in to categories as a 10-year increase in age has previously been shown to be associated with a two times greater risk of hip fracture [110]. As illustrated in **Figure 3.5**, in the case-cohort, the percentage of fractures at the hip, spine and wrist combined (total fractures) for the age categories 39-49 years, 50-59 years, 60-69 years and 70-78 years were 6%, 10%, 12% and 22% in men, respectively, and 5%, 13%, 24% and 41% in women. Older men and women were more likely to suffer a fracture at the hip, spine or wrist (total fracture) over the median follow-up of 12.6 years (*P*<0.001).

Figure 3.5: The effects of age on total fractures in the EPIC-Norfolk case-cohort sample.



Sex-specific chi square tests indicated that the number of fractures differed significantly between age groups in both sexes (P<0.001). n=5012.

Men who were classed as being physically "inactive" or "moderately inactivate" were more likely to experience a fracture than those who were "moderately active" or "active" (percentage fractures: 13% and 15% *vs.* 9% and 10%, respectively, *P*=0.051). Similarly, physically inactive women had higher fracture rates compared to more active women (percentage fractures: 29% *vs.* 16-18%, *P*<0.001). The use of steroid medication was also associated with higher fractures in women (31% *vs.* 20%, *P*=0.004), but not in men. In women, being postmenopausal was associated with much higher fracture rates (27%) compared to being early or late peri-menopausal (10-11%) and being pre-menopausal (5%, all *P*<0.001). Moreover, women who had never used HRT were more likely to suffer a fracture than women who were former and current users (24% *vs.* 16% and 12%, respectively, *P*<0.001). Percentage fractures did not differ for the remaining covariates including family history of osteoporosis, BMI, smoking, dietary calcium intake, and the use of calcium and vitamin D supplements in either sex.

3.6 Associations between intakes of fruit and vegetables with bone health

This chapter also investigated potential associations between dietary intakes of fruit and vegetables with bone health for comparison reasons with the following chapters, which will explore nutrient-bone relationships of predominantly plant-based nutrients.

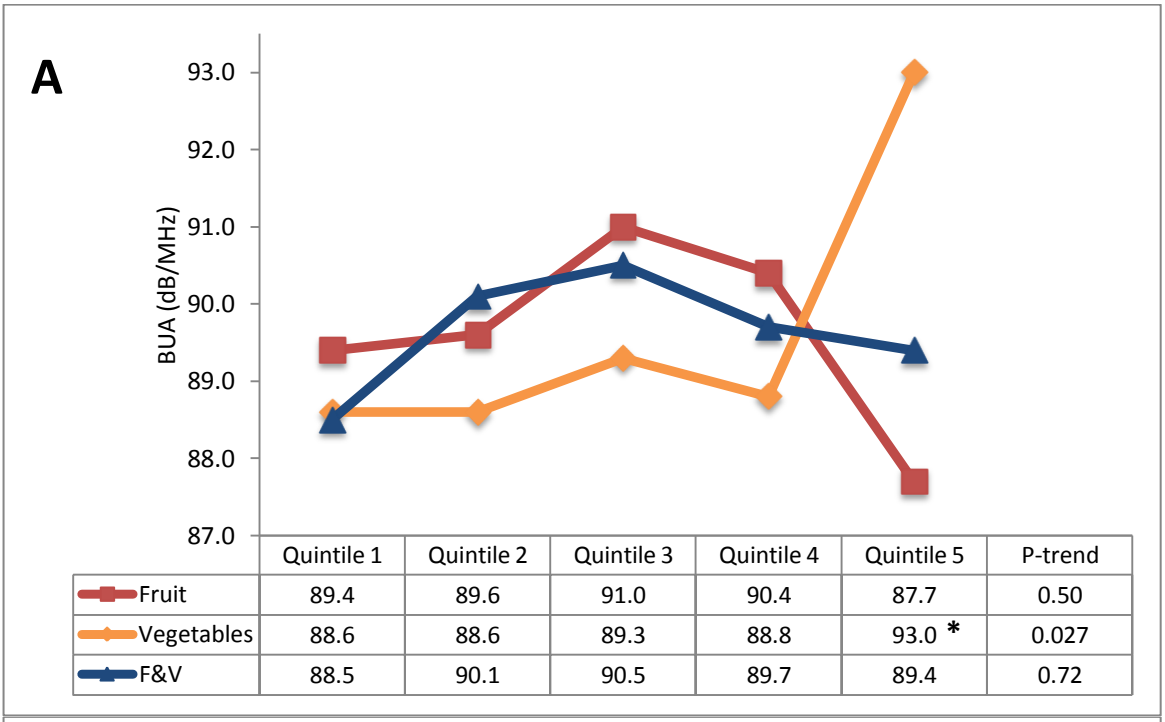
3.6.1 Heel ultrasound

Cross-sectional associations between fruit intake, vegetable intake and the combined total intake of fruit and vegetables (F&V) with heel ultrasound measures are shown in **Figure 3.6** for men and in **Figure 3.7** for women. In men, vegetable intake was significantly positively associated with both BUA (β 0.89 dB/MHz per quintile, *P*-trend=0.027) and VOS (β 1.89 m/s per quintile, *P*-trend=0.037). Moreover, men in the top *vs.* the lowest quintile of vegetable intake had 5.0% higher BUA and 0.7% higher VOS (*P*≤0.014). Intakes of fruit and F&V were not associated with measures of heel ultrasound in men. In women, there were significant positive associations between BUA and intakes of fruit (β 0.74 dB/MHz per quintile, *P*-trend=0.008), vegetables (β 1.13 dB/MHz per quintile, *P*-trend<0.001) and F&V (β 1.10 dB/MHz per quintile, *P*-trend<0.001). Moreover, women in the top *vs.* the lowest quintile of fruit, vegetable and F&V intake had 5.1%, 7.3% and 7.1% higher BUA, respectively (*P*≤0.004). Higher vegetable intake was also significantly associated with 0.4% higher VOS in women (β 1.64 m/s per quintile, *P*-trend=0.017), but there were no associations between fruit and F&V with VOS in women.

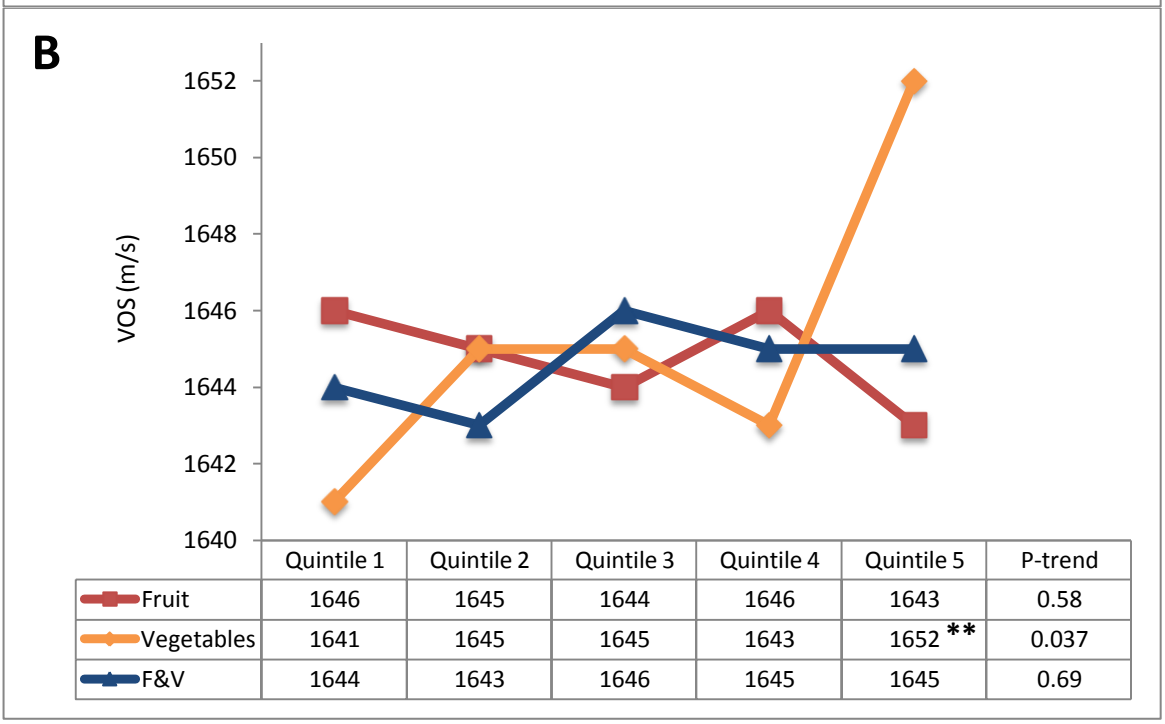
3.6.2 Fracture risk

There were no differences in fruit and vegetable intakes in participants with and without a fracture **(Table 3.4)**. When subjects were grouped into quintiles of intake **(Table 3.5)**, hip fracture risk in men was significantly inversely associated with fruit intake (HR 0.81, 95%CI 0.69-0.96; *P*-trend=0.014) and with F&V intake (HR 0.85, 95%CI 0.72-0.99; *P*-trend=0.043), and those men in quintile 4 compared to quintile 1 had significantly lower hip fracture risk (fruit intake: HR 0.31, 95%CI 0.15-0.65; *P*=0.002; F&V intake: HR 0.43, 95%CI 0.22-0.87; *P*=0.018). There were no associations between fruit and vegetable intakes and the risk of spine or wrist fracture in men. In women, there was a marginally significant association between fruit intake and spine fracture risk (HR 0.86, 95%CI 0.74-1.00; *P*-trend=0.046), and spine fracture risk was significantly lower in women with higher *vs.* lower fruit intakes (Q4 *vs.* Q1: HR 0.54, 95%CI 0.31-0.94; *P*=0.029; Q5 *vs.* Q1: HR 0.56, 95%CI 0.32-0.98; *P*=0.041). There were no associations between fruit and vegetable intakes and the risk of hip or wrist fracture in women.

Figure 3.6: Associations between intakes of fruit and vegetables with mean BUA **(A)** and VOS **(B)** in men.



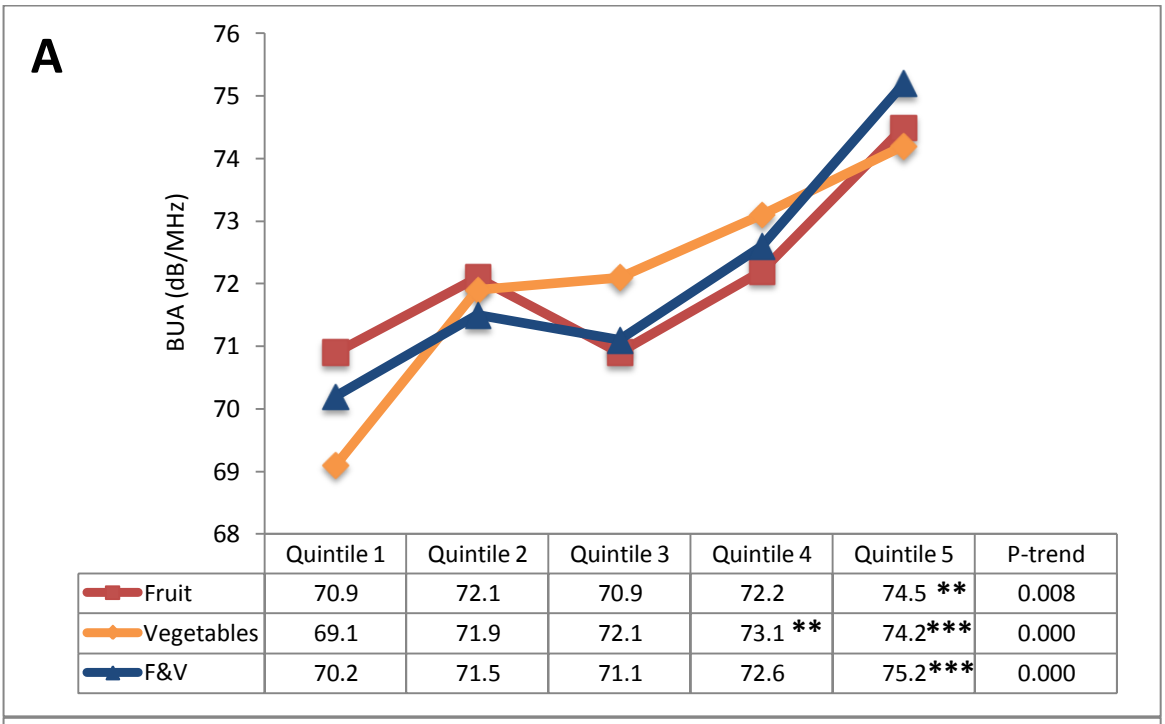
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Fruit	89.4	89.6	91.0	90.4	87.7	0.50
Vegetables	88.6	88.6	89.3	88.8	93.0 *	0.027
F&V	88.5	90.1	90.5	89.7	89.4	0.72



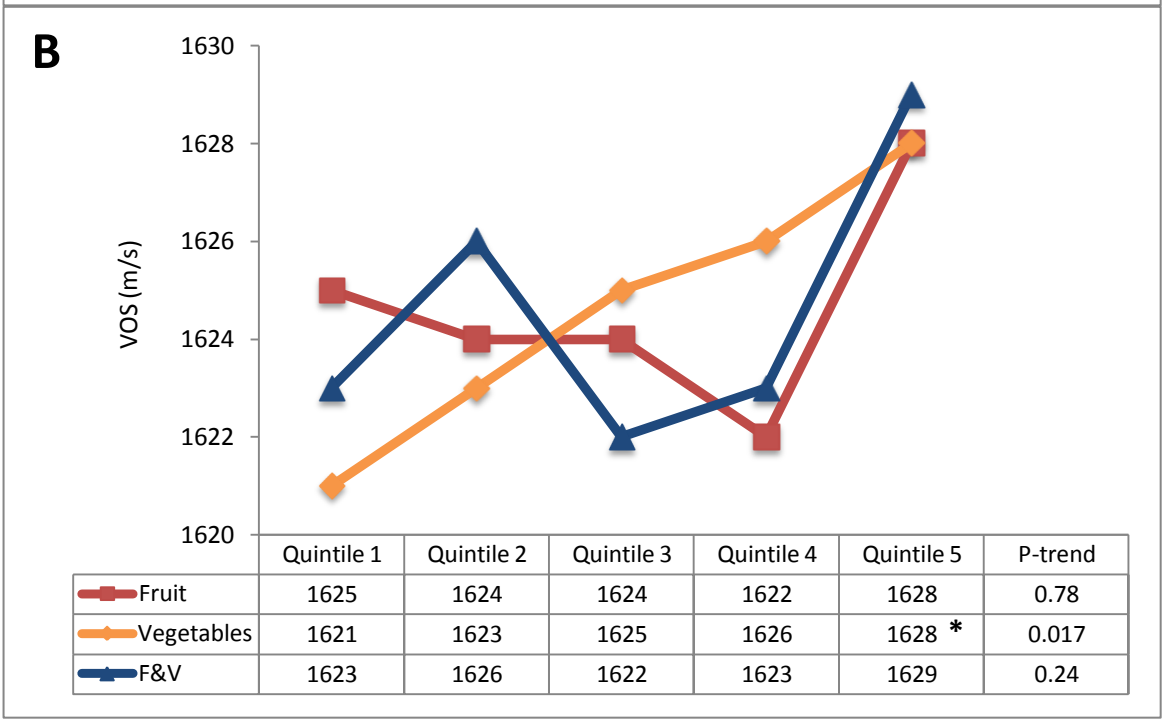
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Fruit	1646	1645	1644	1646	1643	0.58
Vegetables	1641	1645	1645	1643	1652 **	0.037
F&V	1644	1643	1646	1645	1645	0.69

*Mean dietary intakes for quintile 1 and 5 ranged from 17-361 g/d for fruit intake, 32-221 g/d for vegetable intake and 84-521 g/d for the combined fruit and vegetable intake (F&V). Standard error of the mean (SE) was 1.2-1.3 dB/MHz for BUA and 2.8-2.9 m/s for VOS. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05 and **P<0.01. n=968.*

Figure 3.7: Associations between intakes of fruit and vegetables with mean BUA **(A)** and VOS **(B)** in women.



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Fruit	70.9	72.1	70.9	72.2	74.5 **	0.008
Vegetables	69.1	71.9	72.1	73.1 **	74.2***	0.000
F&V	70.2	71.5	71.1	72.6	75.2***	0.000



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Fruit	1625	1624	1624	1622	1628	0.78
Vegetables	1621	1623	1625	1626	1628 *	0.017
F&V	1623	1626	1622	1623	1629	0.24

*Mean dietary intakes for quintile 1 and 5 ranged from 38-384 g/d for fruit intake, 39-221 g/d for vegetable intake and 110-541 g/d for the combined fruit and vegetable intake (F&V). Standard error of the mean (SE) was 0.9 dB/MHz for BUA and 2.1-2.4 m/s for VOS. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05, **P<0.01 and ***P<0.001. n=1360.*

Table 3.4: Fruit and vegetable intakes in subjects with and without a total fracture.

	Subjects without a fracture				Subjects with a fracture				
Dietary intake (g/d)	***n***	**Mean**	**(SD)**	**[Range]**	***n***	**Mean**	**(SD)**	**[Range]**	***P***
Men									
Fruit and vegetables	1709	249.4	(164.2)	[0; 2143]	248	242.2	(155.3)	[0; 1086]	0.52
Fruit	1709	143.1	(131.3)	[0; 1603]	248	137.2	(134.0)	[0; 1013]	0.51
Vegetables	1709	106.3	(73.3)	[0; 626]	248	105.0	(69.9)	[0; 432]	0.80
Women									
Fruit and vegetables	2139	284.2	(167.4)	[0; 2375]	616	284.0	(167.5)	[0; 1135]	0.98
Fruit	2139	174.8	(134.1)	[0; 1624]	616	172.3	(131.0)	[0; 936]	0.68
Vegetables	2139	109.4	(69.3)	[0; 787]	616	111.6	(73.5)	[0; 506]	0.48

Table 3.5: Associations between quintiles of fruit and vegetable intakes and fracture risk.

	Men				Women			
	n **cases/ non-cases**	**Q4 *vs.* Q1**	**Q5 *vs.* Q1**	**Linear trend**	***n*** **cases/ non-cases**	**Q4 *vs.* Q1**	**Q5 *vs.* Q1**	**Linear trend**
Total fracture								
Fruit and vegetables	248/1709	HR 0.85; *P*=0.48 (95%CI 0.55-1.33)	HR 0.74; *P*=0.19 (95%CI 0.47-1.16)	HR 0.94; *P*=0.24 (95%CI 0.85-1.04)	616/2139	HR 1.01; *P*=0.95 (95%CI 0.74-1.37)	HR 0.98; *P*=0.90 (95%CI 0.71-1.35)	HR 1.01; *P*=0.85 (95%CI 0.94-1.08)
Fruit	248/1709	HR 0.73; *P*=0.18 (95%CI 0.47-1.15)	HR 0.66; *P*=0.07 (95%CI 0.42-1.04)	HR 0.91; *P*=0.06 (95%CI 0.82-1.00)	616/2139	HR 0.92; *P*=0.62 (95%CI 0.68-1.26)	HR 0.98; *P*=0.26 (95%CI 0.61-1.15)	HR 0.98; *P*=0.62 (95%CI 0.91-1.06)
Vegetables	248/1709	HR 0.83; *P*=0.39 (95%CI 0.53-1.28)	HR 0.91; *P*=0.68 (95%CI 0.60-1.40)	HR 0.99; *P*=0.90 (95%CI 0.90-1.10)	616/2139	HR 1.00; *P*=1.00 (95%CI 0.73-1.37)	HR 1.01; *P*=0.95 (95%CI 0.74-1.37)	HR 1.02; *P*=0.57 (95%CI 0.95-1.10)
Hip fracture								
Fruit and vegetables	112/1730	**HR 0.43; *P*=0.018 (95%CI 0.22-0.87)**	HR 0.63; *P*=0.16 (95%CI 0.33-1.20)	**HR 0.85; *P*=0.043 (95%CI 0.72-0.99)**	339/2187	HR 1.12; *P*=0.56 (95%CI 0.76-1.66)	HR 0.92; *P*=0.71 (95%CI 0.61-1.40)	HR 1.02; *P*=0.72 (95%CI 0.92-1.12)
Fruit	112/1730	**HR 0.31; *P*=0.002 (95%CI 0.15-0.65)**	HR 0.53; *P*=0.06 (95%CI 0.28-1.02)	**HR 0.81; *P*=0.014 (95%CI 0.69-0.96)**	339/2187	HR 1.07; *P*=0.76 (95%CI 0.71-1.59)	HR 0.92; *P*=0.69 (95%CI 0.61-1.39)	HR 1.03; *P*=0.58 (95%CI 0.94-1.13)
Vegetables	112/1730	HR 0.96; *P*=0.89 (95%CI 0.51-1.79)	HR 0.77; *P*=0.43 (95%CI 0.40-1.49)	HR 1.00; *P*=0.97 (95%CI 0.86-1.16)	339/2187	HR 1.05; *P*=0.83 (95%CI 0.69-1.58)	HR 0.92; *P*=0.69 (95%CI 0.61-1.39)	HR 1.00; *P*=0.94 (95%CI 0.91-1.09)
Spinal fracture								
Fruit and vegetables	78/1730	HR 1.28; *P*=0.49 (95%CI 0.63-2.62)	HR 0.82; *P*=0.61 (95%CI 0.38-1.76)	HR 1.02; *P*=0.82 (95%CI 0.87-1.20)	124/2211	HR 0.68; *P*=0.23 (95%CI 0.37-1.26)	HR 1.06; *P*=0.83 (95%CI 0.60-1.88)	HR 0.93; *P*=0.29 (95%CI 0.80-1.07)
Fruit	78/1730	HR 1.14; *P*=0.70 (95%CI 0.57-2.29)	HR 0.67; *P*=0.30 (95%CI 0.31-1.42)	HR 0.98; *P*=0.78 (95%CI 0.82-1.16)	124/2211	**HR 0.54; *P*=0.029 (95%CI 0.31-0.94)**	**HR 0.56; *P*=0.041 (95%CI 0.32-0.98)**	**HR 0.86; *P*=0.046 (95%CI 0.74-1.00)**
Vegetables	78/1730	HR 0.79; *P*=0.51 (95%CI 0.39-1.58)	HR 0.76; *P*=0.43 (95%CI 0.38-1.50)	HR 0.98; *P*=0.81 (95%CI 0.82-1.17)	124/2211	HR 0.85; *P*=0.58 (95%CI 0.48-1.52)	HR 0.97; *P*=0.92 (95%CI 0.57-1.66)	HR 1.02; *P*=0.81 (95%CI 0.89-1.16)
Wrist fracture								
Fruit and vegetables	70/1736	HR 1.54; *P*=0.28 (95%CI 0.70-3.38)	HR 1.06; *P*=0.90 (95%CI 0.46-2.45)	HR 1.06; *P*=0.46 (95%CI 0.91-1.24)	218/2192	HR 0.85; *P*=0.46 (95%CI 0.55-1.31)	HR 0.81; *P*=0.36 (95%CI 0.52-1.27)	HR 0.98; *P*=0.66 (95%CI 0.88-1.09)
Fruit	70/1736	HR 1.19; *P*=0.69 (95%CI 0.52-2.71)	HR 0.93; *P*=0.87 (95%CI 0.40-2.16)	HR 0.99; *P*=0.91 (95%CI 0.85-1.16)	218/2192	HR 0.76; *P*=0.24 (95%CI 0.48-1.20)	HR 0.76; *P*=0.24 (95%CI 0.48-1.20)	HR 0.94; *P*=0.26 (95%CI 0.84-1.05)
Vegetables	70/1736	HR 0.49; *P*=0.14 (95%CI 0.19-1.26)	HR 1.43; *P*=0.34 (95%CI 0.69-2.95)	HR 1.01; *P*=0.95 (95%CI 0.83-1.22)	218/2192	HR 0.99; *P*=0.96 (95%CI 0.63-1.55)	HR 1.06; *P*=0.80 (95%CI 0.68-1.63)	HR 1.04; *P*=0.42 (95%CI 0.94-1.15)

Values are adjusted Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check.

3.7 Summary

In summary, this case-cohort sample of the EPIC-Norfolk study was a large sub-sample of over 5000 healthy participants aged between 39 and 79 years at baseline, 60% of which were women. This study had a very low proportion of current smokers compared to the general population of England at the time of recruitment. Diet was as expected, with men having a higher total energy intake and subsequent higher intake of most nutrients compared to women. Dietary intakes of both vitamin C and vitamin K_1 were slightly lower in this population compared to the general population, and iron intake was slightly higher, possibly due to the use of different dietary assessment methods.

Participants with a fracture at the hip, spine or wrist compared to those without were significantly older, less active, more likely to take steroid medication, and had a higher total energy intake. Moreover, further preliminary analyses showed that increasing age, a family history of osteoporosis, low BMI, current smoking, low levels of physical activity, the use of steroid medication, and in women, postmenopausal status and no HRT use, were significantly associated with lower heel ultrasound or an increased risk for fracture. These preliminary results highlight the necessity for adjusting for confounding factors, which are associated with measures of heel ultrasound and the risk of fractures in this population, and therefore the analyses of the next chapters included multivariate adjustments.

For comparison reasons with the results from the next chapters, the relationship between fruit and vegetable intake and bone health were determined in preliminary analyses. The results showed that, in cross-sectional analyses, heel ultrasound measures were significantly positively associated with vegetable intake in men and with all measures of fruit and vegetable intake in women. In prospective investigations, intakes of fruit and F&V were significantly inversely associated with hip fracture risk in men, and fruit intake with spine fracture risk in women.

In the following chapters, the role of diet in osteoporosis and fracture prevention in older age will be explored by investiagting dietary relationships with markers of bone health. Then, strategies of dealing with issues arising from the measurement of dietary behaviour will be explored by combining estimates of dietary intake and nutrient status in blood.

CHAPTER 4

VITAMIN K_1 AND BONE HEALTH

Cross-sectional and prospective investigations of vitamin K_1 intake with heel ultrasound and fracture risk

4.1 Abstract

Vitamin K_1 may play a role in reducing bone-related inflammation and it is crucial to the calcium-binding ability of osteocalcin, the most abundant non-collagenous protein in bone. Previous epidemiological studies have shown significant associations between higher dietary vitamin K_1 intakes and markers of blood status with higher bone density and lower risk of hip fracture. However, epidemiological evidence in men is scarce, and data from British cohorts is limited to only one previous study despite population differences in dietary intakes of vitamin K_1. Therefore, this study aimed to explore i) potential cross-sectional associations between dietary vitamin K_1 intake and measures of heel ultrasound and ii) potential prospective associations with fracture risk in a sub-set of the 25,639 EPIC-Norfolk men and women aged 39-79 years at baseline. The results from the present cross-sectional study showed that vitamin K_1 intake was significantly associated with 0.6% higher VOS in men and with 5.5% higher BUA in women. The largest difference in mean vitamin K_1 intake between the highest and lowest group was 133 µg/d in men and 121 µg/d in women, and this is achievable through the usual diet. In the prospective study, vitamin K_1 intake was not a significant predictor of fracture risk at any site in men, although a significant association between higher vitamin K_1 intake and a 53% reduction in spine fracture risk was found in women. The present cross-sectional and prospective investigations addressed previous limitations regarding the scarcity of data in men and from British populations. Our findings highlight the importance green leafy vegetables as the main source of vitamin K_1 in our diet, and these may have significant implications in maintaining higher levels of bone density. Future studies should conduct long-term RCTs to investigate the effects of vitamin K_1 supplementation and fracture risk as this has not been conducted before.

4.2 Introduction

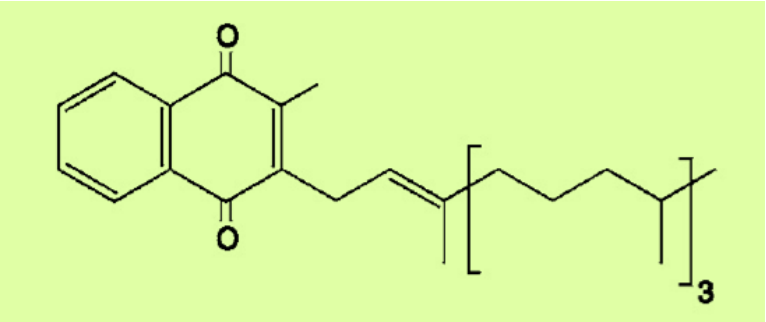
Vitamin K_1 is exclusively found in plant-based foods [214] and has been suggested to play a role in reducing bone-related inflammation [150, 151, 231], a process associated with upregulated bone resorption [232]. Furthermore, it acts as a cofactor in the γ-carboxylation of osteocalcin, the most abundant non-collagenous protein in bone, which is crucial for its ability to bind calcium [11]. The proportion of osteocalcin in blood that is not carboxylated (undercarboxylated osteocalcin, ucOC) is a sensitive marker of vitamin K status and has previously been shown to be an independent predictor of low BMD and fracture risk [233-237].

Vitamin K, a class of fat-soluble organic compounds, may be classified into two naturally occurring groups (K_1 and K_2) according to the synthesising medium, but also exist as a number of synthetic forms [214, 238-240]. The underlying mechanisms of vitamin K in various processes in the body may not be dependent on its molecular forms because the latter share a common methylated naphthoquinone ring structure. Their differences lie in the side chain which is attached to this ring. Vitamin K_1 has a phytyl side chain and vitamin K_2 has varying numbers of isoprenoid residues depending on its molecular form. As the methylated naphthoquinone ring is thought to be the functional group of vitamin K, the different forms are likely to act similarly.

A number of vitamins and medications are thought to alter normal vitamin K absorption and metabolism. For example, very high vitamin A intakes may interfere with vitamin K absorption [241]; whereas high vitamin E intakes may alter vitamin K metabolism [242] and lead to a decrease in vitamin K status [243, 244]. Moreover, anticoagulants, such as warfarin, have been shown to inhibit vitamin K epoxide reductases [245], and aspirin may interfere with vitamin K metabolism via the inhibition of quinone reductases [246]. The use of aspirin has also been associated with reduced fracture healing in rats [247]. Following intestinal absorption, the transport of vitamin K to the liver, where it is stored, occurs in the form of triglyceride-rich lipoproteins [248]. Unlike those of other fat-soluble vitamins, the onset of vitamin K deficiency may be rapid as vitamin K stores are quickly depleted [249].

Despite our current knowledge of vitamin K absorption, metabolism and storage, investigations of its requirements in humans are limited, and thus a UK RNI for vitamin K has not been established yet. However, dietary intakes of 1 μg/kg/d in adults have been proposed as safe intakes for the UK population [225]. This reflects average daily intakes of vitamin K of 60-90 μg/d as estimated in several British and American studies [160, 250, 251] and is slightly lower than the US Adequate Intake recommendations of 90-120 μg/d [252]. Mean dietary intakes appear to be lower in British populations (men: 70 μg/d; women: 61 μg/d) [160] compared to those in the US (men: 143 μg/d; women: 163 μg/d; or median 163 μg/d) [158, 159], possibly in part due to differences in dietary assessments.

Figure 4.1: The structure of vitamin K_1 (phylloquinone).



Adapted from Berkner (2005) [(253)].

Vitamin K_1 **(Figure 4.1)**, also referred to as phylloquinone, is exclusively synthesised by green plants and plays a key role in photosynthesis. For this reason, it is predominantly found in the photosynthesis-active parts of plants such as spinach and kale, and in much smaller quantities in the roots and fruits of plants as well as some vegetable oils and margarines [(214)]. The bioavailability of vitamin K_1 is relatively low because the vitamin is tightly bound to the chloroplast membrane in the photosynthetic-active parts of plants, thereby limiting intestinal absorption [(254)]. Moreover, due to the fat-soluble properties, its absorption in the human intestine is dependent on the solubilisation and emulsification of its lipophilic compounds by pancreatic enzymes and bile salts.

In the last few decades, a range of other vitamin K-dependent proteins have been identified, including osteocalcin and matrix Gla protein, which are involved in bone metabolism [(255)]. Hence, a potential role for vitamin K in osteoporosis and fracture prevention was proposed and epidemiologic evidence confirmed this with reports of significant positive associations between, for example, higher vitamin K_1 intakes and reduced fracture risk [(154, 158, 159)]. However, epidemiological evidence in men is scarce, and data from British cohorts is limited despite population differences in dietary intakes of vitamin K_1 [(158-160)].

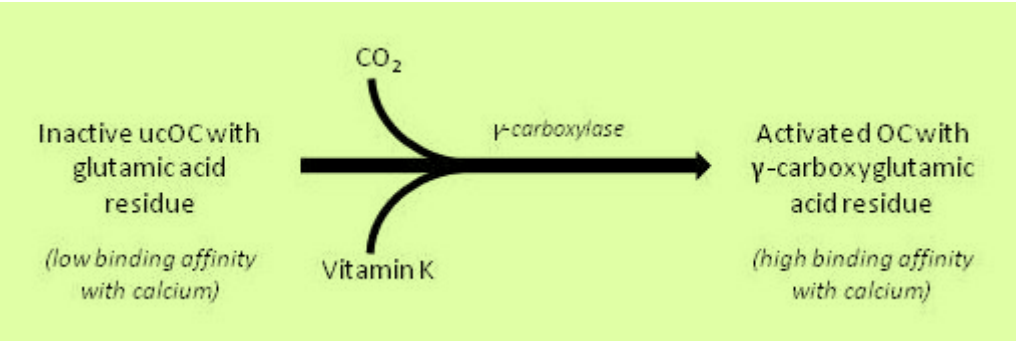
4.2.1 The potential role of vitamin K_1 in bone health

Since the structures of vitamin K_1 and K_2 are very similar and because a still undefined proportion of vitamin K_1 is converted into K_2 in the body, previous experimental studies have mainly evaluated and reported the underlying mechanisms on bone for compounds with vitamin K-activity. Hence, the following section will focus on the latter with respect to bone rather than the specific mechanisms of action of vitamin K_1 alone.

4.2.1.1 The γ-carboxylation of osteocalcin

To date, the vitamin K-dependent γ-carboxylation of osteocalcin and its subsequent ability to bind calcium is the primary underlying mechanism for the hypothesised beneficial effects of vitamin K on bone [(11)]. Osteocalcin is the most abundant non-collagenous protein in bone, accounting for around 10-20% of the non-collagenous proteins and approximately 1% of all bone protein [(256)]. It is synthesised by osteoblasts during bone formation [(257, 258)] and is mainly involved in the mineralisation of chondrocytes, found in bone cartilage. Osteocalcin requires calcium in order to maintain its structural integrity, with each osteocalcin molecule binding five calcium ions via its three glutamate residues. However, this process requires γ-carboxylation [(259)], and this is vitamin K-dependent [(253)] **(Figure 4.2)**. Vitamin K acts as a cofactor in the post-translational γ-carboxylation of glutamic acid to γ-carboxyglutamic acid in osteocalcin. The latter has a strong binding affinity with calcium, and this enables a protein-calcium-phopholipid interaction [(260)]. In the absence of vitamin K, γ-carboxylation does not occur, and osteocalcin lacks structural integrity and remains biologically inactive. The fraction of osteocalcin, which is unable to bind to calcium due to the absence of the γ-carboxylation process, is referred to as ucOC. Serum levels of ucOC, when expressed as a fraction of total osteocalcin, are considered to be a sensitive marker of vitamin K status [(261)], and high circulating ucOC levels may be an indicator of low dietary intakes of vitamin K [(262)].

Figure 4.2: The γ-carboxylation of glutamic acid residues in osteocalcin.



Abbreviations: OC, osteocalcin; ucOC, undercarboxylated osteocalcin.
Adapted from Tie et al. (2011) [(263)].

Besides osteocalcin, matrix Gla protein and protein S have also been identified as vitamin K-dependent proteins of bone matrix [(264, 265)]; however, their role in bone health is not yet fully understood. Matrix Gla protein contains five Gla residues and is found in bone and cartilage in high levels [(266, 267)]. Protein S has also been shown to be present in bone matrix and is synthesised by osteoblasts [(268)].

4.2.1.2 The modulation of pro-inflammatory cytokines

Another potential role of vitamin K in bone health relates to its ability to reduce bone-related inflammation. For example, one animal study in rats found that following the induction of inflammation, vitamin K_1 supplementation suppressed inflammation [150]. It has been suggested that the underlying mechanisms for this might be that vitamin K_1 has the ability to suppress IL-6 expression [150, 151]. IL-6 is a pro-inflammatory cytokine which stimulates osteoclastogenesis; hence it is involved in bone resorption [232]. In fact, in a recent cross-sectional study of 662 older participants, those subjects in the top quartile compared to the bottom quartile of serum phylloquinone concentration had significantly lower levels of circulating IL-6 (adjusted mean ± SEM: 1.22±0.07 pmol/l *vs.* 1.45±0.07 pmol/l, *P*-trend<0.01) [231]. This suggests that vitamin K may be beneficial to bone by reducing inflammation, which has previously been shown to partly contribute to the development of osteoporosis [49, 269], through the down-regulation of IL-6 expression in osteoclastogenesis.

4.2.2 Associations between vitamin K_1 and bone health in previous studies

To date, there is evidence from RCTs and epidemiological studies for a potential beneficial role of vitamin K_1 in osteoporosis and fracture risk, although the evidence is inconsistent. In these studies, bone health was investigated as i) fracture incidence as the best indicator of bone health, ii) BMD using DXA as the gold standard method or other measurements including quantitative ultrasound, and iii) biochemical markers of bone formation including BSALP, OC, PINP and PICP, as well as markers of bone resorption including PYD, DPD, CTx and NTx. The following section will review previously published studies which investigated potential associations between vitamin K_1 and bone health. RCTs as the best indicator of causality will be discussed first, and this will be followed by observational studies in hierarchical order of decreasing ability to determine causality.

4.2.2.1 Randomised controlled trials

RCTs are the best studies for inferring causality and for determining which factors influence disease. Thus, they are the gold standard as they limit both selection biases and confounding. A recent meta-analysis of RCTs by Fang *et al.* [270] assessed the impact of vitamin K supplementation on BMD at the spine and femoral neck measured via DXA by extrapolating data from 16 intervention studies in 20-599 participants published between 1999 and 2010. They reported beneficial effects of vitamin K supplementation on BMD at the spine but not the femoral neck (percentage change in BMD: 1.27% at the spine, *P*=0.002; and 0.17% at the femoral neck, *P*=0.38). However, these findings were no longer significant after the exclusion of low-quality studies which had been classified as ≤3 points on the Jadad scale, a scale with a

maximum score of five assessing the quality of randomisation, blinding, and withdrawals and drop-outs [271]. Moreover, the results of this meta-analysis included both phylloquinone (K_1) and menaquinones (K_2) studies, with the majority of studies supplementing with vitamin K_2 and only five of sixteen studies investigating vitamin K_1. A summary of those vitamin K_1 studies can be found in **Appendix 1, Table A1.1**.

Additional subgroup analysis by Fang *et al.* [270], using data from the two vitamin K_1 studies that focused on absolute effects of lumbar spine BMD [272, 273], did not find a significant beneficial effect. Differences in population characteristics (age: 68 *vs.* 25-50 years; sex: men and postmenopausal women *vs.* pre- and postmenopausal women), duration of study (36 *vs.* 6 months), supplementation dose (500 *vs.* 600 µg of phylloquinone) and concurrent treatment (calcium and vitamin D *vs.* no concurrent treatment) may explain why the subgroup analysis as part of the meta-analysis did not find a significant overall effect of vitamin K_1 supplementation on spine BMD. The inclusion of previous study outcomes at other bone sites in addition to those of the spine may have given a different result. This is because a well-designed RCT in healthy women reported that those receiving a vitamin K_1 supplement for three years had up to 1.7% less BMD loss at the femoral neck compared to those that did not receive vitamin K_1 [153]. Moreover, there is also evidence from more recent data published after the meta-analysis and a summary of these studies can be found in **Appendix 1, Table A1.2**. For example, one study also reported beneficial effects of vitamin K_1 on different BMD sites [152]. In this study, vitamin K_1 supplementation of 100 µg/d for 12 months resulted in 1.1% and 1.35% higher BMD at the whole body and the spine respectively compared to baseline. In contrast, the control group experienced whole body and spinal BMD losses of 0.1% and 2.9%, respectively. The supplementation dose of 100 µg/d used in this study would be easily achievable in the diet, as mean dietary vitamin K_1 intakes of 97-171 µg/d have previously been reported for different population groups in large scale epidemiological studies [154, 155, 157].

Furthermore, the meta-analysis by Fang *et al.* only focused on the effects of vitamin K_1 on changes in BMD [270]. However, a large number of studies, including those already included in the meta-analysis, additionally investigated the effects of vitamin K_1 on a range of markers of bone metabolism. Although findings were contradictory, intervention studies consistently reported beneficial effects of vitamin K_1 supplementation on levels of undercarboxylated osteocalcin (ucOC), an indicator of low vitamin K status [261]. Vitamin K_1 supplementation, ranging from 80 to 1000 µg/d, resulted in a decrease in ucOC concentrations of 31-50% compared to baseline values; and decreases in ucOC levels of 40-68% were reported in comparison to the control groups [274-277]. Where different doses were given, a dose-response relationship was observed with higher vitamin K_1 supplementation doses resulting in greater reduction of ucOC concentrations [276]. Similarly, studies reporting results for undercarboxylated osteocalcin as a percentage of total osteocalcin (%ucOC) showed significant decreases in %ucOC of 33-54%

compared to baseline values [152, 272, 275, 278]. Other markers of bone turnover shown to be beneficially affected by vitamin K_1 include BSALP [279], PINP [277], CTx [277] and DPD [152, 279]. For example, BSALP increased by 30% and PINP, CTx and DPD decreased by 15%, 30% and 10%, respectively, compared to baseline as a result of vitamin K_1 supplementation (80-10000 µg/d) for 4-29 months. However, a number of studies did not find any significant effects of vitamin K_1 supplementation on these bone turnover markers [153, 274-276, 280], possibly due to differences in study duration, doses and composition of the vitamin K_1 supplements, and the overall health of the study population. There is also great controversy regarding the effects of vitamin K_1 on OC concentrations. Previous intervention studies have reported an increase [276], decrease [274, 277, 279, 280] or no significant changes [152, 153, 272, 273, 278] in OC concentrations as a result of vitamin K_1 supplementation. It has previously been suggested that the use of different antibodies during OC analysis may be an explanation for these discrepancies, resulting from the antibodies' differing affinity for the carboxylated form of osteocalcin [261].

Although a number of RCTs have investigated the effects of vitamin K_1 on changes in BMD or markers of bone turnover, most RCTs were conducted in female populations only. Only two of the eleven identified RCTs included men in their study populations [272, 278]; and findings indicated beneficial effects of vitamin K_1 supplementation on %ucOC, but not on changes in BMD. Hence, the effects of vitamin K_1 supplementation on bone remain to be explored further in men.

Higher ucOC concentrations have previously been determined as an independent risk factor for osteoporotic fractures (OR 2.0, 95%CI 1.2-3.2) [235]. Considering that all RCTs discussed in this literature review have reported beneficial effects of vitamin K_1 on ucOC concentrations, it is likely that vitamin K_1 would also have beneficial effects on fracture risk, possibly through the reduction of ucOC concentrations. In fact, there is epidemiological evidence that high ucOC concentrations may be a marker of fracture risk [235]. However, to date, no experimental studies have investigated the effects of vitamin K_1 on fracture risk and thus further research is needed to confirm this hypothesis.

There is evidence to suggest that vitamin K_1 supplementation may be beneficial to bone health. Previous RCTs have reported beneficial effects of on BMD as well as on various markers of bone turnover, particularly ucOC as an indicator of vitamin K status. However, previous findings remain inconsistent, possibly due to differences in follow-up, study durations, study populations and supplementation doses. Furthermore, the long-term effects of vitamin K_1 supplementation on bone health are not yet fully understood as studies were of short duration (3 years or less) and data on fracture risk are still lacking.

4.2.2.2 Prospective studies

Prospective cohort studies may be used to investigate the aetiology of a disease as the exposure is measured prior to the condition occurring, making data less prone to recall bias than case-control studies. Furthermore, as cases and controls are drawn from the same population, there is less selection bias. A summary table of prospective studies investigating potential associations between vitamin K_1 and bone density can be found in **Appendix 1, Table A1.3**.

Previous prospective studies on dietary intakes of the nutrient have reported no associations. The only four published studies to date were undertaken in US, Chinese, Spanish and Danish populations of 200-2217 older participants. They used measurements of BMD [158, 281, 282] or ultrasound [156]; however, no significant associations between vitamin K_1 intake and any one of these bone measures were found. Potential explanations for this might be issues around small sample sizes, high mean vitamin K_1 intakes with limited variance in intakes, and a short follow-up period. For example, two prospective studies investigated associations in small cohorts of under 1000 participants [156, 158]. Moreover, in one study, vitamin K_1 intakes were very high [156], with mean intakes of 334 μg/d in men and 300 μg/d in women compared to 59-82 μg/d as estimated for the general US population [250]. This may have been due to the use of a FFQ which tends to overestimate dietary intakes including fruit and vegetable intake [202, 204] and subsequently introduces measurement error. To date, no prospective studies have investigated vitamin K_1 and bone relationships in British populations, despite the latter having lower dietary intakes than US populations [158-160], as previously discussed.

In contrast, prospective and longitudinal studies have almost consistently shown that higher vitamin K_1 intakes are associated with a lower risk for hip fracture [157-159]. For example, in 888 US men and women (mean age = 75 years), those with median dietary vitamin K_1 intakes of 254 μg/d had a up to 65% lower risk of hip fracture than those with intakes of 56 μg/d after a 7-year follow-up period [158]. Similarly, every 10 μg/d increment in dietary vitamin K_1 intake was significantly associated with a 2% risk reduction in hip fracture in 2807 Norwegian men and women (mean age = 72 years) [157]. Only one prospective study in 2944 Chinese men and women (mean age = 74 years) did not find any associations [283], possibly due to the population's high dietary intakes of vitamin K_1 (range: 155-362 μg/d in men and 162-408 μg/d in women), which may limited the study's ability to detect a potential discrimination in fractures at the extreme ends of vitamin K_1 intake. Prospective studies also reported a link between vitamin K_1 status and fractures. For example, the relative risk of vertebral fractures was 3.58 (95%CI, 3.26-3.93) for subjects with lower plasma K_1 levels than the median (2.67 nmol/l) compared to those with plasma vitamin K_1 levels above the median in a population of 379 Japanese women (mean age = 63 years) [284]. Another prospective study in less than 200 French women aged 70-97 years also showed that serum ucOC concentrations were significantly higher in subjects who sustained a hip fracture compared to those who did not (ucOC: 1.47 ng/ml *vs.* 0.89 ng/ml, $P<0.05$) [236]. In

contrast, another prospective study did not find an association between ucOC levels and the incidence of vertebral fractures [284]; however, this study did not adjust for any confounding factors. Future prospective studies investigating potential associations between ucOC and vertebral fractures should therefore adjust for relevant covariates.

4.2.2.3 Case-control studies

Case-control studies are used to examine specific exposures as potential risk factors of a disease in people with and without the condition. The exposures preceded the disease outcome, and thus case-control studies are prone to recall bias where case subjects tend to have a better recollection of specific exposures than the controls. Moreover, selection bias is an issue as both the exposure and the disease outcome are pre-defined. Results from seven previous case-control studies provide further evidence for a link between dietary vitamin K_1 intake and bone health **(Appendix 1, Table A1.4)**. Studies have consistently reported that cases with osteopenia, low BMD or hip fractures had significantly lower serum or plasma vitamin K_1 levels than controls (cases: serum 0.24-0.41 ng/ml; plasma 0.27-0.46 ng/ml; controls: serum 0.55-0.64 ng/ml; plasma 0.39-0.77 ng/ml) [285-289]. Fracture cases were also shown to have significantly lower BMD at different measurement sites compared to controls (cases: hip BMD 0.758 g/cm^2; spine BMD 0.981 g/cm^2; controls: hip BMD 0.795 g/cm^2; spine BMD 1.033 g/cm^2) [281]. To date, no previous study compared dietary intakes of vitamin K_1 between cases and controls. Two case-control studies investigated associations between vitamin K_1 and the risk of hip fracture [281, 285]. The results showed that serum vitamin K_1 levels were a significant predictor of hip fracture risk (OR 0.07, 95%CI 0.02-0.32; *P*=0.001) [285], although hip fracture risk according to dietary intakes of vitamin K_1 did not differ between cases and controls [281], possibly due to the young age and narrow age range of the studied population (48-52 years).

4.2.2.4 Cross-sectional studies

Cross-sectional studies are used to report the prevalence of a disease in a defined population at a specific point in time. Whether the exposure predated the disease or not cannot be determined. To date, positive associations between vitamin K_1 intake or blood concentrations and bone health have also been shown in a number of cross-sectional studies **(Appendix 1, Table A1.5)**. For example, higher dietary vitamin K_1 intakes were associated with higher BMD *(no effect sizes shown)* [154, 155]; and lower intakes were significantly associated with a higher risk of having low BMD (*P*-trend=0.007) [157]. Moreover, every 100 µg/d increment in dietary vitamin K_1 intake was associated with an 0.96 dB/MHz increase in BUA and a 1.13 m/s increase in SOS [156]. For blood concentrations, each 1 nmol/l increase in plasma concentrations of phylloquinone in men was significantly associated with an increase in BUA of 1.13 dB/MHz and in SOS of 1.6 m/s [290].

Moreover, women with higher circulating levels of ucOC had significantly lower BMD at multiple sites (for example spine BMD: β±SE -0.008±0.003 g/cm^2, *P*=0.008) [233]. However, the potentially beneficial associations between vitamin K_1 and BMD were not reported in all cross-sectional studies, possibly in part due to small sample sizes (<900 men and women) [158] and differences in dietary assessment methods (4dDD or 7dDD *vs.* FFQ) [158, 281, 290]. Although markers of bone formation have not previously been shown to be associated with intakes of vitamin K_1, possibly partly due to small sample sizes [154, 156], free pyridinoline cross-links relative to creatinine (PYD/Cr) and free deoxypyridinoline cross-links relative to creatinine (DPD/Cr), which are markers of bone resorption, were found to be significantly associated with intakes of the vitamin [154]. In this instance, those women with lower vitamin K_1 intakes (59 µg/d *vs.* 162 µg/d) had higher levels of these bone resorption markers (PYD/Cr: 5.4 *vs.* 5.1 nmol/mmol; DPD/Cr: 19.8 *vs.* 18.7 nmol/mmol).

Interestingly, previous cross-sectional studies undertaken in men and women have failed to show an association between vitamin K_1 intake and bone health separately in men. Although one study found potentially beneficial effects, the reported findings represented data from a combined sample of men and women [156]. In contrast, studies that performed their analyses separately for men and women did not report any significant associations in men [155, 157, 158]. Sex-specific differences in the effects of vitamin K on bone have been suggested as a potential explanation for these findings, although previous metabolic studies investigating the effects of vitamin K depletion and supplementation on vitamin K status and markers of bone turnover did not support such a hypothesis [155].

To date, a much larger body of cross-sectional evidence regarding potential associations between vitamin K_1 intake or blood concentrations and bone health exists for women compared to men, and thus more studies in men are needed. Moreover, data from British populations is scarce, with only one previous cross-sectional study investigating potential associations between vitamin K_1 intake and BMD and markers of bone turnover in a British cohort of only early post-menopausal women [154], despite previously reported differences in dietary vitamin K_1 intakes between populations [158-160].

4.2.2.5 Conclusion of previously published studies

To date, eleven RCTs have examined the effects of vitamin K_1 supplementation on changes in BMD and markers of bone turnover, and reported contradictory findings. Differences in study populations, study durations as well as vitamin K_1 supplementation doses and composition may have affected the diversity of study results. Future studies should consider longer duration of supplementation when looking at changes in BMD as positive effects of vitamin K_1 have previously been found after supplementation periods of 12-36 months. Furthermore, long-term data on the effects of vitamin K_1 supplementation on fracture risk is lacking.

Previous epidemiological studies have shown a positive association between dietary intakes of vitamin K_1 as well as markers of vitamin K_1 status and bone health. However, findings have been contradictory, possibly due to differences in sample sizes, age ranges, sex, mean vitamin K_1 intakes and dietary intake measures. Moreover, epidemiological evidence in men is scarce, and data from British cohorts is limited to only one previous study despite population differences in dietary intakes of vitamin K_1. This limits the extent to which the potential relationship between vitamin K_1 on bone can be interpreted and understood, and thus more epidemiological studies in the general population are needed to address some of these limitations.

4.2.3 Chapter aims and objectives

In order to address some of these limitations, this chapter aimed to:

i) Investigate potential cross-sectional associations between dietary vitamin K_1 intake estimated from a 7dDD and the heel ultrasound parameters BUA and VOS.

ii) Examine potential prospective associations between dietary vitamin K_1 intake and the risk of fracture at the hip, spine and wrist in a British population of men and women aged between 39 and 79 years at baseline.

These investigations will provide more evidence for potential associations in men in particular, but also for British populations which tend to have lower dietary intakes of vitamin K_1 than US populations [158-160]. Moreover, this study will be based on using more accurate dietary intake information (7dDD) compared to the majority of previous studies (FFQs), as FFQs tend to overestimate dietary intakes including those of fruit and vegetables [202, 204]. It was hypothesised that dietary intakes of vitamin K_1 are positively associated with measures of bone density and inversely associated with the risk of fracture.

4.3 Methods

As discussed in Chapter 2 (page 40), two studies were performed on a randomly selected sample of men and women of the EPIC-Norfolk prospective cohort study. Briefly, the cross-sectional study of heel ultrasound was based on a random sub-cohort of 4000 participants who had attended the first health check, and the prospective investigations of fracture risk were based on a case-cohort design using the same subset of 4000 participants and a set of 1502 participants who had experienced a fracture up to 31st March 2009. For both types of studies, analyses using dietary intakes of vitamin K_1 estimated from a 7dDD as the predictor variable were performed using quintiles of intake. In the first study, multiple regression with multivariate adjustment was used to assess the cross-sectional relation of quintiles of vitamin K_1 intake to BUA and VOS. Both BUA and VOS are measures of heel ultrasound, but BUA is an indicator of the structural organisation of bone, whereas VOS determines bone stiffness [63]. In the second study, Kaplan-Meier survival curves alongside log-rank tests of equality were computed to evaluate differences in crude total fracture incidence over the median 12.6-year follow-up between the quintile groups. Then, Prentice-weighted Cox proportional hazard ratios [221] were used to assess the prospective relations of quintiles of vitamin K_1 to fracture risk for three important fracture sites (hip, spine and wrist) as well as total fractures. In both studies, potential associations between the top two quintiles referent to the lowest quintile of vitamin K_1 intake were investigated. As previously discussed, all analyses were stratified by sex and adjusted for relevant confounders using an unadjusted and two multivariate models (Chapter 2, page 49). The final model included age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. Moreover, the procedures for dealing with missing data and the number of exclusions in each study are described in detail in Chapter 3 (pages 55-57). For the purpose of this chapter, participants were also excluded from the cross-sectional study of heel ultrasound if they had missing data at the second health check for the use of aspirin medication (*n*=326), leaving 2002 participants (854 men and 1148 women) for analysis. In the fracture risk study, there were no participants with missing aspirin data from the first health check. However, three men with very high dietary intakes of vitamin K_1 (>550 µg/d), which had been estimated from only one completed day of the 7dDD, were also excluded from the subsequent prospective analyses, thus leaving 4709 (1954 men and 2755 women) in the fracture study. In order to be able to put the present findings in to context, in this chapter, a sample size calculation was performed post-hoc for the associations between BUA and VOS with the top quintile referent to the lowest quintile of vitamin K_1 intake. The present chapter was chosen because the exclusion of participants with missing aspirin data resulted in the smallest dataset compared to the other nutrient chapters. Moreover, the sample size calculations were

undertaken for the heel ultrasound study as this was a smaller dataset than the fracture study. The sample size calculations for BUA and VOS in men and women were based on the method by Charan & Biswas [291] which accounts for the epidemiological nature of the present study:

Sample size = (2*(SD^2) * ((Z[a/2]+Z[b])^2) / (d^2)

Where: Z(a/2) = a constant at type I error of 5% = 1.96
Z(b) = a constant at 80% power = 0.842
d = effect size (Q5 *versus* Q1).

4.4 Results

4.4.1 Descriptive statistics stratified by quintiles of vitamin K_1 intake

The characteristics of the 4709 participants of the EPIC-Norfolk the case-cohort sample (59% women) stratified by quintiles of vitamin K_1 intake are shown in **Table 4.1**. In the 1954 men, mean±SD dietary vitamin K_1 intake for the quintile groups were as follows: Q1 43.5±9.2 μg/d, Q2 64.1±5.2 μg/d, Q3 82.5±5.6 μg/d, Q4 105.7±8.4 μg/d and Q5 172.5±59.3 μg/d. The mean dietary vitamin K_1 intake for each quintile was slightly lower in the 2755 women than in men: Q1 38.4±9.4 μg/d, Q2 59.1±4.8 μg/d, Q3 76.3±5.7 μg/d, Q4 99.9±8.4 μg/d and Q5 164.1±63.3 μg/d. Age did not differ across quintiles of vitamin K_1 intake in both sexes, but there was a small but significant decrease in BMI with increasing vitamin K_1 intakes in women (P=0.004). Men in the upper quintiles were more likely to have a family history of osteoporosis (P=0.031), and were less likely to be current smokers (P=0.023). The latter was also found in women (P=0.024), and women in the upper quintiles were also more active compared to those in the lowest quintile (P<0.001). Moreover, the use of aspirin medication significantly decreased across quintiles of vitamin K_1 intake and the use of calcium and vitamin D supplements significantly increased in women only (P≤0.020).

4.4.2 Associations between vitamin K_1 intake and heel ultrasound

Associations between the bone density parameters BUA (in dB/MHz) and VOS (in m/s) with dietary vitamin K_1 intake are presented in **Figure 4.3**. The results are discussed in detail below. Briefly, we found that dietary vitamin K_1 intake was significantly positively associated with VOS in men and with BUA in women.

In the 854 men and 1148 women, dietary vitamin K_1 intake did not correlate with measurements of heel ultrasound (men: BUA and VOS r=0.06, P>0.05; women: BUA r=0.03 and VOS r=0.00, P>0.05). In linear regression analyses in men, higher vitamin K_1 intake was marginally significantly associated with higher VOS (β 2.0±1.0 m/s per quintile, P-trend=0.045), even after adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, aspirin medication, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. Moreover, men in the highest quintile of vitamin K_1 intake had 0.56 % higher adjusted VOS compared to those men with the lowest dietary intakes (P=0.039). No such associations were found with BUA in men. In women, dietary intakes of vitamin K_1 were significantly and positively associated with BUA following the adjustment for confounding factors (β 0.81±0.31 dB/MHz per quintile, P-trend=0.009). Moreover, women in the top quintile had 5.5% higher adjusted BUA compared to those women in the lowest quintile of intake (P=0.004), and the 3.8% difference between quintile 4 and quintile 1 was almost significant (P=0.052). There were no associations between vitamin K_1 intake and VOS in women.

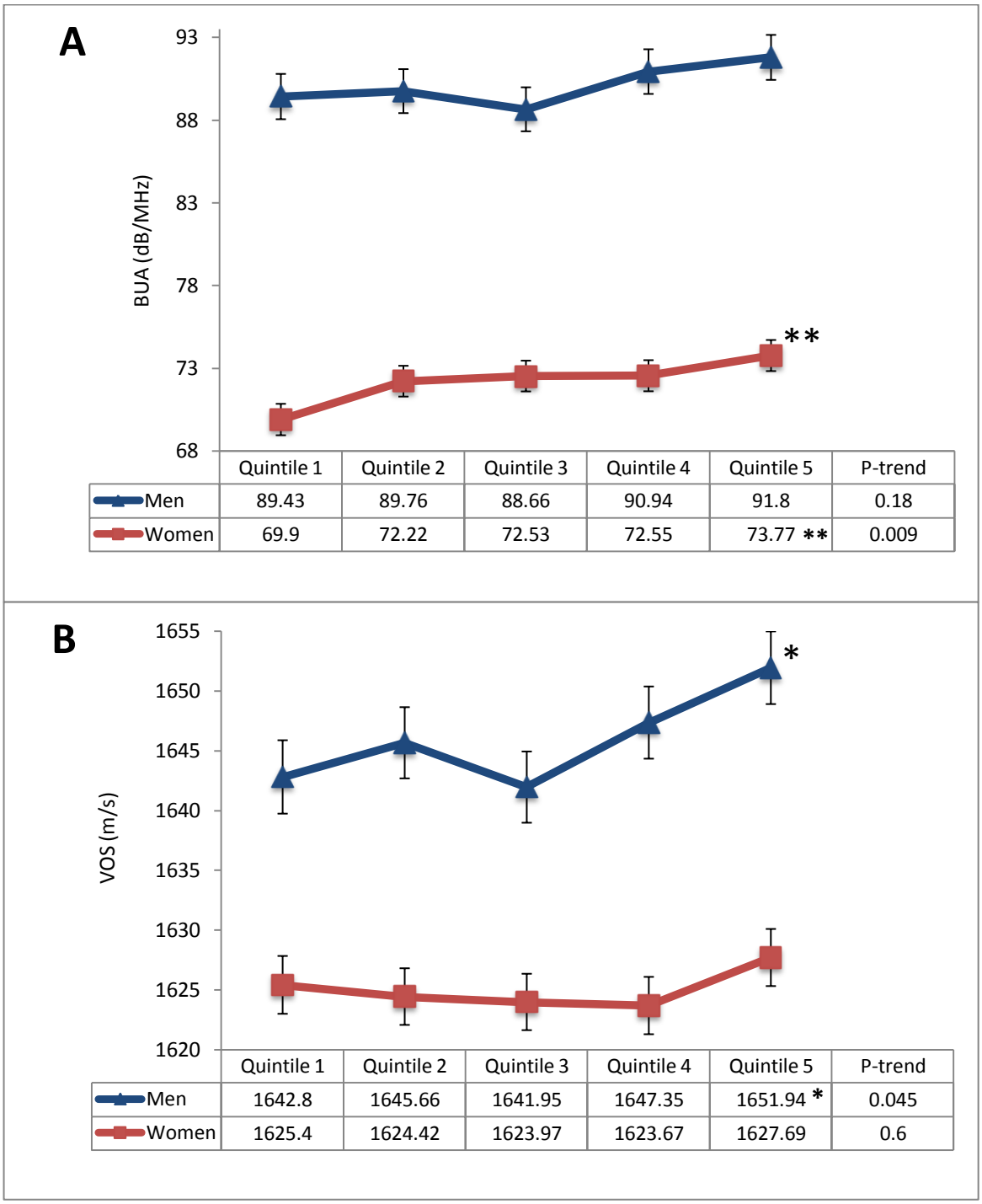
The post-hoc sample size calculations showed that associations between vitamin K_1 intake and heel ultrasound were significant despite small quintile sample sizes. For example, the association between quintile 5 referent to quintile 1 and VOS in men was significant with a quintile sample size of 170 participants despite the calculation of 303 subjects to detect this significant difference at 80% power. Similarly in women, 284 participants per quintile would have been required to detect the difference in extreme quintiles of vitamin K_1 intake and BUA, but these associations were detected with a smaller number of women per quintile in the present study (*n*=230). As previously discussed, there were no associations between vitamin K_1 intake and BUA in men and VOS in women, possibly due to small quintile sample sizes. The number of participants required for each quintile to detect these effect sizes was 825 men and 4749 women compared to 170 men and 230 women in the present study, respectively.

Table 4.1: Baseline characteristics of the 1954 men and 2755 women of the EPIC-Norfolk case-cohort by quintiles of vitamin K_1 intake.

	Men											Women										
Vitamin K_1 intake (µg/d)	**Quintile 1 10.3 – 55.2**		**Quintile 2 55.3 – 73.3**		**Quintile 3 73.4 – 92.3**		**Quintile 4 92.4 – 120.6**		**Quintile 5 120.7 - 459.2**		**P-trend**	**Quintile 1 5.0 – 50.7**		**Quintile 2 50.8 – 67.0**		**Quintile 3 67.1 – 86.3**		**Quintile 4 86.4 – 116.4**		**Quintile 5 116.5 – 611.5**		**P-trend**
	n = 391		***n = 391***		***n = 391***		***n = 391***		***n = 390***			***n = 551***		***n = 551***		***n = 551***		***n = 551***		***n = 551***		
Mean (SD)																						
Age (years)	59.5	(9.8)	59.5	(9.6)	59.6	(9.4)	59.5	(9.5)	60.7	(9.5)	*P*=0.10	60.5	(10.0)	59.4	(9.7)	59.5	(9.9)	59.4	(9.2)	60.2	(8.9)	*P*=0.65
BMI (kg/m^2)	26.7	(3.4)	26.5	(3.3)	26.5	(3.2)	26.5	(3.3)	26.3	(3.4)	*P*=0.23	26.8	(4.7)	26.0	(4.3)	26.0	(4.3)	26.1	(4.0)	25.9	(4.3)	*P*=0.004
n (%)																						
Menopausal Status																						*P*=0.031
Pre-mp	-	-	-	-	-	-	-	-	-	-		81	(14.7)	93	(16.9)	93	(16.9)	79	(14.4)	68	(12.3)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		15	(2.7)	28	(5.1)	31	(5.6)	26	(4.7)	27	(4.9)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		102	(18.5)	80	(14.5)	85	(15.4)	106	(19.2)	75	(13.6)	
Post-mp	-	-	-	-	-	-	-	-	-	-		353	(64.1)	350	(63.5)	342	(62.1)	340	(61.7)	381	(69.2)	
HRT																						*P*=0.048
Current User	-	-	-	-	-	-	-	-	-	-		107	(19.4)	93	(16.9)	94	(17.1)	97	(17.6)	81	(14.7)	
Former User	-	-	-	-	-	-	-	-	-	-		74	(13.4)	46	(8.4)	60	(10.9)	75	(13.6)	69	(12.5)	
Never Used	-	-	-	-	-	-	-	-	-	-		370	(67.2)	412	(74.7)	397	(72.0)	379	(68.8)	401	(72.8)	
Smoking											*P*=0.023											*P*=0.024
Current smoker	64	(16.4)	55	(14.1)	47	(12.0)	41	(10.5)	31	(8.0)		88	(16.0)	69	(12.5)	67	(12.2)	54	(9.8)	65	(11.8)	
Former smoker	213	(54.5)	205	(52.4)	214	(54.7)	228	(58.3)	220	(56.4)		180	(32.7)	155	(28.1)	189	(34.3)	177	(32.1)	189	(34.3)	
Never smoked	114	(29.1)	131	(33.5)	130	(33.3)	122	(31.2)	139	(35.6)		283	(51.3)	327	(59.4)	295	(53.5)	320	(58.1)	297	(53.9)	
Physical activity											*P*=0.68											*P*<0.001
Inactive	137	(35.0)	114	(29.2)	127	(32.5)	118	(30.2)	116	(29.7)		228	(41.4)	195	(35.4)	175	(31.7)	163	(29.6)	147	(26.7)	
Mod. inactive	94	(24.1)	100	(25.6)	92	(23.5)	93	(23.8)	92	(23.6)		172	(31.2)	158	(28.7)	168	(30.5)	188	(34.1)	191	(34.6)	
Mod. active	90	(23.0)	88	(22.5)	83	(21.2)	91	(23.3)	83	(21.3)		87	(15.8)	123	(22.3)	125	(22.7)	112	(20.3)	130	(23.6)	
Active	70	(17.9)	89	(22.8)	89	(22.8)	89	(22.7)	99	(25.4)		64	(11.6)	75	(13.6)	83	(15.1)	88	(16.0)	83	(15.1)	
Family history of OP	8	(2.1)	11	(2.8)	5	(1.3)	14	(3.6)	19	(4.9)	*P*=0.031	29	(5.3)	32	(5.8)	24	(4.4)	35	(6.4)	34	(6.2)	*P*=0.61
Steroids	15	(3.8)	10	(2.6)	13	(3.3)	14	(3.6)	16	(4.1)	*P*=0.80	25	(4.5)	21	(3.8)	28	(5.1)	20	(3.6)	20	(3.6)	*P*=0.68
Aspirin	32	(8.2)	48	(12.3)	34	(8.7)	48	(12.3)	34	(8.7)	*P*=0.12	47	(8.5)	34	(6.2)	27	(4.9)	23	(4.2)	28	(5.1)	*P*=0.020
Calcium supp.	7	(1.8)	6	(1.5)	3	(0.8)	5	(1.3)	4	(1.0)	*P*=0.73	19	(3.5)	24	(4.4)	29	(5.3)	42	(7.6)	41	(7.4)	*P*=0.006
Vitamin D supp.	71	(18.2)	88	(22.5)	93	(23.8)	82	(21.0)	96	(24.6)	*P*=0.20	143	(26.0)	180	(32.7)	160	(29.0)	193	(35.0)	199	(36.1)	*P*=0.001

Values are means (standard deviations) or numbers (frequencies). Abbreviations: Mp, menopausal; Family history of OP, family history of osteoporosis; Supp., supplements.

Figure 4.3: Associations between vitamin K_1 intake and mean BUA **(A)** and VOS **(B)**.



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Men	89.43	89.76	88.66	90.94	91.8	0.18
Women	69.9	72.22	72.53	72.55	73.77 **	0.009

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Men	1642.8	1645.66	1641.95	1647.35	1651.94 *	0.045
Women	1625.4	1624.42	1623.97	1623.67	1627.69	0.6

*Mean vitamin K_1 intake for quintile 1 and 5 ranged from 45.9-178.8 µg/d in men and 42.8-163.7 µg/d in women. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05 and **P<0.01. n=854 men and n=1148 women.*

4.4.3 Associations between vitamin K_1 intake and fracture risk

In the 4709 case-cohort sample of EPIC-Norfolk participants, there were 112 hip fractures, 77 spine fractures and 70 wrist fractures in men, and 339 hip fractures, 124 spine fractures and 218 wrist fractures in women. In the case-cohort that investigated participants with a fracture at any of these three fracture sites (total fracture), there were 247 and 616 fractures in men and women, respectively. The results of the calculation of hazard ratios of fracture risk according to dietary vitamin K_1 intake are discussed below in detail. Briefly, vitamin K_1 was not associated with fracture risk at any site in either sex, except for a significant inverse association between women with higher compared to lower vitamin K_1 intakes and spine fracture risk.

4.4.3.1 Vitamin K_1 intake in participants with or without a fracture

Mean dietary vitamin K_1 intake did not differ significantly between participants who had experienced a total fracture and those who stayed free from fractures over the median 12.6-year follow-up **(Table 4.2)**.

4.4.3.1 Vitamin K_1 intake and fracture risk

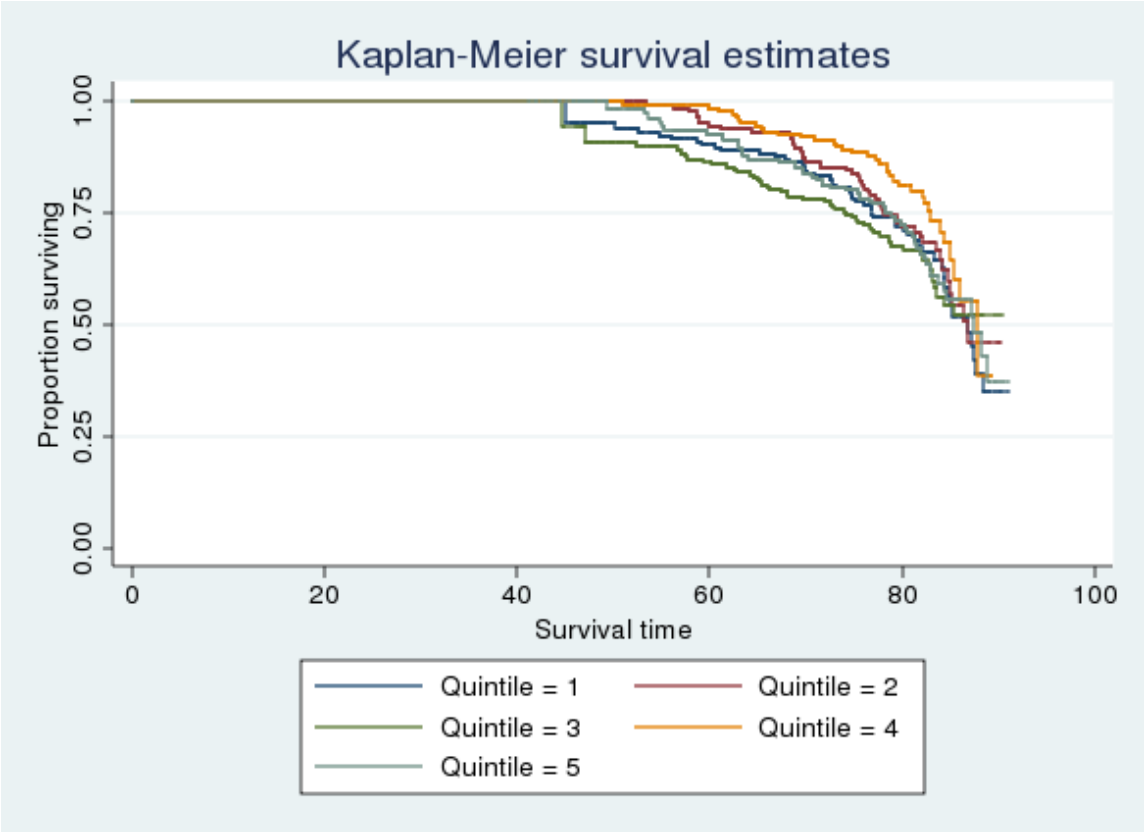
In men, the Kaplan Meier plot showed that there was both overlap and cross-over between the five quintiles of dietary vitamin K_1 intake and no one quintile diverged significantly from the others **(Figure 4.4)**. In concordance with these findings, the results from the Prentice-weighted Cox proportional hazard ratios showed that dietary vitamin K_1 intake was not associated with fracture risk at any site in men **(Table 4.3)**, even after adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroid medication or aspirin medication, energy intake, dietary calcium intake and the use of calcium and vitamin D supplements.

In women, there was both overlap and cross-over between the five quintiles of dietary vitamin K_1 intake **(Figure 4.5)**, but the log-rank test for equality showed that total fracture incidence differed between the five quintile groups (*P*=0.038). The results from the Prentice-weighted Cox proportional hazard ratios showed that dietary vitamin K_1 intake was not associated with fracture risk at any site **(Table 4.4)**. However, there was a significant 53% reduction in spine fracture risk in women in quintile 4 compared to quintile 1 of vitamin K_1 intake (HR 0.47, 95%CI 0.24-0.91; P=0.026), and this was significant before and after the adjustment for important confounding factors.

Table 4.2: Differences in vitamin K_1 intake between participants with or without a total fracture.

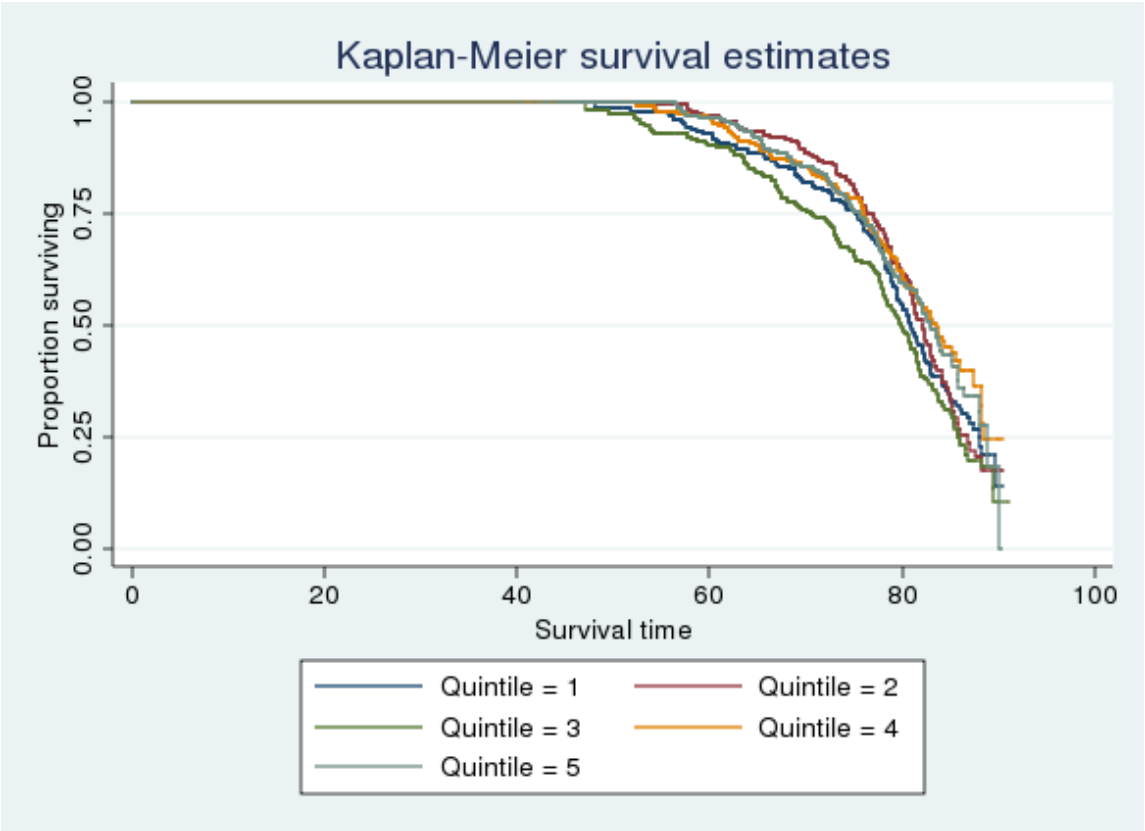
	Vitamin K_1 intake (µg/d)								
	in subjects without a fracture				**in subjects with a fracture**				
	n	**Mean**	**(SD)**	**[Range]**	***n***	**Mean**	**(SD)**	**[Range]**	***P***
Men	1707	93.7	(52.3)	[10.3; 459.2]	247	93.2	(50.8)	[17.2; 354.1]	0.88
Women	2139	88.1	(51.9)	[5.0; 611.5]	616	85.8	(53.0)	[7.1; 533.5]	0.35

Figure 4.4: Kaplan-Meier plot of total fractures by quintiles of vitamin K_1 intake in men.



There were no significant differences between the quintile groups of vitamin K_1 intake according to the log-rank test for equality (P=0.52). n=1954.

Figure 4.5: Kaplan-Meier plot of total fractures by quintiles of vitamin K_1 intake in women.



The quintile groups of vitamin K_1 intake differed significantly according to the log-rank test for equality (P=0.038). n=2755.

Table 4.3: Associations between vitamin K_1 intake and fracture risk in men of the EPIC-Norfolk case-cohort.

		Dietary vitamin K_1 intake (µg/d)								
		Quintile 1		**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		10.3 – 55.2		**55.3 – 73.3**		**73.4 – 92.3**		**92.4 – 120.6**		**120.7 - 459.2**	
		n = 391		***n = 391***		***n = 391***		***n = 391***		***n = 390***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[51]		[51]		[52]		[37]		[56]	
	Unadjusted	1.00	1.00	(0.66-1.54)	0.95	(0.62-1.46)	0.73	(0.47-1.16)	1.05	(0.69-1.59)	*P*=0.74
	Model 1	1.00	1.02	(0.66-1.58)	0.97	(0.63-1.49)	0.75	(0.47-1.19)	1.06	(0.69-1.63)	*P*=0.79
	Model 2	1.00	1.00	(0.64-1.56)	0.93	(0.60-1.44)	0.71	(0.44-1.14)	1.02	(0.66-1.57)	*P*=0.62
Hip fracture	[Events]	[23]		[23]		[23]		[15]		[28]	
	Unadjusted	1.00	0.99	(0.54-1.84)	0.90	(0.48-1.67)	0.67	(0.34-1.34)	1.09	(0.60-1.99)	*P*=0.90
	Model 1	1.00	1.01	(0.52-1.95)	0.91	(0.48-1.73)	0.70	(0.35-1.42)	1.11	(0.60-2.03)	*P*=0.95
	Model 2	1.00	1.03	(0.53-2.01)	0.89	(0.46-1.72)	0.70	(0.34-1.46)	1.19	(0.63-2.22)	*P*=0.93
Spinal fracture	[Events]	[18]		[15]		[16]		[12]		[16]	
	Unadjusted	1.00	0.84	(0.42-1.70)	0.82	(0.41-1.65)	0.69	(0.33-1.46)	0.81	(0.41-1.61)	*P*=0.46
	Model 1	1.00	0.82	(0.40-1.70)	0.83	(0.41-1.68)	0.68	(0.32-1.44)	0.80	(0.39-1.61)	*P*=0.45
	Model 2	1.00	0.79	(0.38-1.65)	0.80	(0.39-1.64)	0.63	(0.29-1.38)	0.73	(0.36-1.50)	*P*=0.34
Wrist fracture	[Events]	[12]		[14]		[15]		[10]		[19]	
	Unadjusted	1.00	1.19	(0.55-2.60)	1.23	(0.57-2.65)	0.86	(0.37-2.00)	1.65	(0.80-3.41)	*P*=0.36
	Model 1	1.00	1.23	(0.56-2.71)	1.23	(0.57-2.67)	0.88	(0.37-2.07)	1.73	(0.83-3.57)	*P*=0.32
	Model 2	1.00	1.11	(0.50-2.47)	1.07	(0.49-2.33)	0.73	(0.31-1.71)	1.40	(0.66-2.96)	*P*=0.67

Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. No significant differences between the two upper quintiles referent to the lowest quintile. Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids or aspirin. Model 2 additionally adjusted for energy intake, dietary calcium intake, use of calcium supplements and use of vitamin D supplements. n 1954 for total fracture, n 1840 for hip fracture, n 1805 for spine fracture, n 1804 for wrist fracture.

Table 4.4: Associations between vitamin K_1 intake and fracture risk in women of the EPIC-Norfolk case-cohort.

		Dietary vitamin K_1 intake (µg/d)									
		Quintile 1	**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**		
		5.0 – 50.7	**50.8 – 67.0**		**67.1 – 86.3**		**86.4 – 116.4**		**116.5 – 611.5**		
		n = 551	***n = 551***		***n = 551***		***n = 551***		***n = 551***		
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[135]		[115]		[144]		[106]		[116]	
	Unadjusted	1.00	0.92	(0.68-1.25)	1.16	(0.86-1.56)	0.80	(0.59-1.09)	0.87	(0.64-1.18)	*P*=0.24
	Model 1	1.00	0.90	(0.66-1.22)	1.16	(0.86-1.57)	0.81	(0.59-1.11)	0.90	(0.66-1.23)	*P*=0.41
	Model 2	1.00	0.91	(0.67-1.25)	1.19	(0.88-1.61)	0.85	(0.62-1.17)	0.94	(0.69-1.29)	*P*=0.63
Hip fracture	[Events]	[75]		[59]		[79]		[60]		[66]	
	Unadjusted	1.00	0.89	(0.60-1.33)	1.14	(0.78-1.66)	0.88	(0.59-1.31)	0.95	(0.64-1.39)	*P*=0.78
	Model 1	1.00	0.89	(0.60-1.33)	1.16	(0.79-1.70)	0.89	(0.59-1.34)	1.00	(0.67-1.49)	*P*=0.99
	Model 2	1.00	0.91	(0.60-1.36)	1.18	(0.80-1.74)	0.93	(0.61-1.42)	1.04	(0.69-1.56)	*P*=0.80
Spinal fracture	[Events]	[33]		[19]		[29]		[14]		[29]	
	Unadjusted	1.00	0.62	(0.34-1.12)	0.89	(0.53-1.50)	**0.44**	**(0.23-0.84)***	0.92	(0.55-1.55)	*P*=0.51
	Model 1	1.00	0.61	(0.34-1.12)	0.88	(0.52-1.49)	**0.46**	**(0.24-0.88)***	0.93	(0.55-1.57)	*P*=0.57
	Model 2	1.00	0.63	(0.34-1.14)	0.89	(0.52-1.50)	**0.47**	**(0.24-0.91)***	0.94	(0.55-1.58)	*P*=0.64
Wrist fracture	[Events]	[47]		[43]		[53]		[38]		[37]	
	Unadjusted	1.00	0.97	(0.63-1.51)	1.16	(0.76-1.76)	0.82	(0.52-1.28)	0.76	(0.49-1.20)	*P*=0.17
	Model 1	1.00	0.90	(0.58-1.41)	1.13	(0.74-1.74)	0.80	(0.51-1.27)	0.75	(0.47-1.20)	*P*=0.20
	Model 2	1.00	0.91	(0.58-1.44)	1.16	(0.76-1.77)	0.85	(0.53-1.35)	0.78	(0.49-1.25)	*P*=0.30

Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. No significant differences between the two upper quintiles referent to the lowest quintile. Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids or aspirin, menopausal status and HRT. Model 2 additionally adjusted for energy intake, dietary calcium intake, use of calcium supplements and use of vitamin D supplements. n 2755 for total fracture, n 2526 for hip fracture, n 2335 for spine fracture, n 2410 for wrist fracture.

4.5 Discussion

These epidemiological investigations of potential associations between vitamin K_1 intake and measures of heel ultrasound and fracture risk addressed some of the previous limitations in the literature, including the scarcity of epidemiological evidence in men and the limited availability of data from British populations, which tend to have lower dietary intakes of vitamin K_1 than US populations [158-160]. Following multivariate adjustment, the results from the cross-sectional study of heel ultrasound showed that dietary vitamin K_1 intake was significantly positively associated with VOS in men and with BUA in women. In the prospective investigations of fracture risk, dietary vitamin K_1 intake was not associated with the risk of fracture at any site in men, although a significant association between higher intakes (quintile 4 *vs.* quintile 1) and a reduction in spine fracture risk was found in women.

4.5.1 Heel ultrasound

In the cross-sectional study, higher dietary intakes of vitamin K_1 were significantly associated with higher heel ultrasound measurements. In men, there was a marginal linear relationship between vitamin K_1 and VOS, with men with the highest dietary intakes (122-459 μg/d) having 0.6% higher VOS compared to those men with the lowest intakes (19-58 μg/d). A similar linear relationship was also found in women, where those with the highest intakes (121-578 μg/d) had 5.5% higher BUA compared to those with the lowest intakes (12-55 μg/d). The difference in mean vitamin K_1 intake between the extreme quintile groups was approximately 133 μg/d in men and 121 μg/d in women. This may be equivalent to the consumption of one 100g bag of mixed lettuce leaves [214] which demonstrates that the intakes associated with these findings are achievable through the habitual diet. The cross-sectional findings of a positive association between dietary vitamin K_1 intake and measures of heel ultrasound potentially reflect the important role of the nutrient in bone health. Vitamin K_1 has been suggested to play a role in reducing bone-related inflammation [150, 151, 231], a process associated with upregulated bone resorption [232]. Moreover, it acts as a cofactor in the γ-carboxylation of osteocalcin, the most abundant non-collagenous protein in bone, which is crucial for its ability to bind calcium [11]. Furthermore, vitamin K_1 may also be one explanatory factor for the positive associations found between fruit and vegetable intakes and bone health in this cohort. Vitamin K_1 is exclusively found in plant-based foods such as green leafy vegetables [214], and positive associations between intakes of fruit and vegetables were reported in previous epidemiological studies [90, 131-134], although the underlying mechanisms are not fully established yet. Preliminary analyses in this cohort presented in Chapter 3 (pages 69-71) showed that men in the top *vs.* the lowest quintile of vegetable intake had 0.7% higher VOS (compared to 0.6% for vitamin K_1) and women had 7.3% higher BUA (compared to 5.5% for vitamin K_1).

In the present study, percentage differences in VOS were much smaller than those of BUA, possibly due to the scale differences between these two bone parameters. However, one previous study has shown that their relative fracture risk implications are very similar [66]. The present magnitude of effect of BUA in women (5.5%) is in agreement with the literature, as effect sizes of 3.6-5.8% for hip and lumbar spine BMD have previously been found with higher intakes of vitamin K_1 [155]. Moreover, the present findings were also comparable to effect sizes previously reported for other bone-related dietary factors including intakes of potassium (3-4%) and magnesium (3%) [133]. In previous studies, associations between vitamin K_1 and VOS were either not significant or reported differently to the present study [156, 290], hence the magnitude of effect could not be compared.

Interestingly, in the present study, vitamin K_1 intakes were associated with VOS in men and BUA in women. Potential reasons for this sex difference are currently not known. However, there is evidence regarding the independent heritability of the two ultrasound parameters [77], and both measures have also been shown to be independently associated with osteoporotic fractures [21, 69, 70].

4.5.2 Fracture risk

To date, a number of prospective studies have investigated potential associations between intakes of vitamin K_1 and fracture risk, and those have predominantly found a reduction in fracture risk with higher dietary intakes after 7-10 years of follow-up [157-159]. However, to the best of my knowledge, there is no data from British populations despite population differences in dietary intakes, and hence our investigations are novel. In the present prospective study of 4709 British men and women, there were no significant associations between vitamin K_1 intake and fracture risk at multiple sites after the 12.6-year follow-up, although women with higher dietary intakes (86-116 µg/d) compared to those with the lowest intakes (5-51 µg/d) had 53% lower spine fracture risk. The present findings are in agreement with only one previous study which also reported a lack of association between dietary intakes of vitamin K_1 and hip and non-vertebral fracture risk in an older Chinese population of 2944 men and women **[283]**. In contrast, in line with vitamin K_1 being exclusively found in plant-based foods [214], intakes of fruit and vegetables were significantly inversely associated with fracture risk at the hip in men and the spine in women in this cohort (Chapter 3, pages 69-71). This may suggest that the potentially beneficial role of fruit and vegetables in reducing fracture risk [134] may not be related to dietary intakes of vitamin K_1. Nevertheless, the lack of association in the present study may be related to the habitual consumption of the nutrient in British populations [160], including the EPIC-Norfolk population. Currently, habitual vitamin K_1 intakes are not routinely estimated for the UK population by large scale studies such as the National Diet and Nutrition Survey [292]. However, around 60% of participants in this study had dietary intakes equal to or above the UK safe intake

level of 1 μg/kg/d [225], suggesting that it was a relatively healthy population. Despite these adequate intakes, dietary intakes from US populations tend to be higher [158, 159]; and those prospective studies, which reported significant inverse associations between vitamin K_1 intake and fracture risk, were based on US population groups with much higher mean dietary intakes than the present study. For example, mean vitamin K_1 intakes in the present study were 98 μg/d in men and 91 μg/d in women, and those were much lower than dietary intakes in the study by Feskanich *et al.* (median intake: 163 μg/d) [159] and Booth *et al.* (mean intake: men: 143 μg/d; women: 163 μg/d) [158]. Thus, dietary vitamin K_1 intakes in the present study may have been too low to detect any significant associations with fracture risk. Another explanation may be the use of different dietary assessment methods. In our study, vitamin K_1 intake was estimated from a 7dDD, whereas those studies mentioned above were based on FFQs. There is evidence to suggest that FFQs tend to overestimate fruit and vegetable intake [202, 204], and potentially also vitamin K_1 as the main food source of this nutrient are green leafy vegetables.

4.5.3 Strengths and limitations

The present epidemiological investigations had a number of potential strengths over previous studies. The inclusion of both men and women in the study design addressed previous limitations regarding the scarcity of data in men. Moreover, the EPIC-Norfolk cohort provided more evidence for British populations, where data availability is limited despite population differences in dietary vitamin K_1 intakes [158-160]. For example, no prospective study had previously investigated potential associations between vitamin K_1 intake and fracture risk in a British population; and our cohort had a greater age range than the only British study which investigated cross-sectional associations with BMD in women only [154]. Therefore, our cross-sectional findings are applicable to a wider UK population. The present studies also investigated both cross-sectional and prospective associations in the same population using different bone health measures, including heel ultrasound and fracture risk, in contrast to most previous studies which investigated only one of these parameters per population. Furthermore, the post-hoc sample size calculations showed that the significant associations between vitamin K_1 intake and heel ultrasound reached statistical significance despite the quintile sample sizes being smaller than the estimated required sample sizes, indicating the robustness of these associations. Nonetheless, vitamin K_1 intake was not consistently associated with all heel ultrasound parameters in men and women, and this may have been due to small sample sizes which were not large enough to detect smaller effect sizes between extreme quintiles. Further limitations of the present investigations include the cross-sectional study design of the heel ultrasound analyses which only examined relations with diet for a single point in time. The positive associations reported for VOS in men and BUA in women suggest that there was a relation between dietary intakes of vitamin K_1 and measures of heel ultrasound, but conclusions

about the influence of vitamin K_1 on bone health cannot be drawn. Similarly, the prospective study design of the fracture risk analyses was limited by the inability to identify possible secular changes in dietary vitamin K_1 intakes over the follow-up period and subsequent exposure misclassification, as data were only available from the 7dDDs taken at baseline. Moreover, the fracture data had been obtained from hospital admissions which are most likely underestimated for spine fractures due to a large absence in their clinical attention and radiologic detection [168, 293, 294]. This may have reduced the power of the present study to detect the associations between vitamin K_1 intake and spine fracture risk. Although multivariate adjustment models were applied in the analyses, a number of other relevant confounders previously associated with bone health, including sunlight exposure [295], were not measured as part of the EPIC-Norfolk study. Furthermore, residual confounding may have occurred despite the adjustment for covariates and may have resulted in bias in exposure effect estimates.

4.6 Conclusion

The present cross-sectional investigations in EPIC-Norfolk participants found that higher dietary intakes of vitamin K_1 were significantly associated with 0.6% higher VOS in men and 5.5% higher BUA in women. These differences in bone health between those with low and high vitamin K_1 intakes may have important implications for the development of fractures in the long term, although in our prospective investigations, we did not find an association with fracture risk in either sex, possibly due to lower dietary intakes of the nutrient in this cohort compared to previous studies reporting significant effects. The present study provides novel prospective data from a British cohort on fracture risk and vitamin K_1 intake, and addresses a number of limitations of previous cross-sectional studies including the limited availability of data in men. Future epidemiological studies should investigate the association between vitamin K_1 and bone health in other British cohorts in order to address the scarcity of data and enhance our current understanding of potential population differences. Moreover, future studies should conduct RCTs to investigate the effects of vitamin K_1 supplementation and fracture risk, as this has not been conducted before.

CHAPTER 5

VITAMIN C AND BONE HEALTH

Cross-sectional and prospective associations between vitamin C intake and plasma status with heel ultrasound and fracture risk

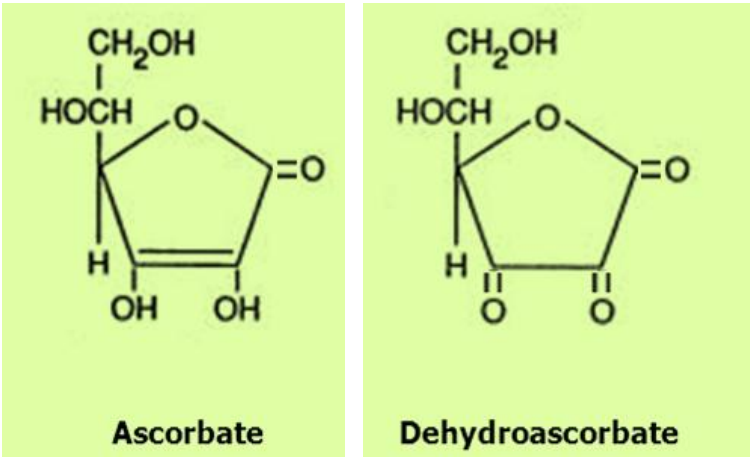
5.1 Abstract

Vitamin C plays a crucial role in bone collagen synthesis and may mediate osteoclastogenesis and osteoblastogenesis. Previous epidemiological studies have shown positive associations between dietary and supplemental intake of vitamin C and bone density, and inverse associations with bone loss and fracture risk. However, studies on blood vitamin C status are scarce as most studies used estimates of vitamin C intake, predominantly from FFQs. Moreover, there is only limited data in men and in British populations, and associations with fracture risk as the clinical endpoint of osteoporosis are underinvestigated. Therefore, this study aimed to explore i) potential cross-sectional associations between dietary intakes and plasma concentrations of vitamin C with measures of heel ultrasound and ii) potential prospective associations between vitamin C intake and plasma status with the risk of fractures in a sub-set of the 25,639 EPIC-Norfolk men and women aged 39-79 years at baseline. The results from the cross-sectional study showed that dietary intakes and total intakes (diet and supplements combined) of vitamin C were significantly associated with 0.6% higher VOS in men and with up to 4.2% higher BUA in women. The largest difference in mean dietary vitamin C intake between the highest and lowest group was 136 mg/d in both men and women, and this is achievable through the usual diet. In the prospective study, higher intakes of vitamin C were significantly associated with 48% lower total fracture risk (hip, spine and wrist fractures combined) in men after a median follow-up of 12.6 years. Moreover, higher plasma concentrations of vitamin C were significantly associated 74%, 65% and 52% lower fracture risk for spine, hip and total fractures, respectively, in men. Vitamin C intake or plasma status was not a significant predictor of fractures in women. This study provides novel prospective data on fracture risk in a British population, and the cross-sectional investigations addressed previous limitations with regards to scarcity of data in men and for vitamin C plasma status. The present findings highlight the importance of fruits and vegetables in our diet, being the main sources of vitamin C, for both short- and long-term bone health. Future studies should consider RCTs which will investigate the effects of vitamin C supplementation on indicators of bone health as this has not been conducted before.

5.2 Introduction

Scurvy, the clinical manifestation of vitamin C deficiency, was first described in the 17th and 18th centuries in sailors on long-haul ocean journeys. However, it was the discovery of vitamin C in the early 20th century and subsequent animal studies that lead to the suggestion of a link between vitamin C and collagen synthesis. Scurvy is associated with wounds and fractures that fail to heal and it was established that this resulted from impaired collagen formation in vitamin C deficiency [296]. Collagen is an essential component of bone tissue with around 98% of the organic phase of bone being comprised of type I collagen [13]. Vitamin C is important for adequate collagen formation via the hydroxylation of prolyl and lysyl residues [7-10]. More recently, many cell and animal studies reported that vitamin C may also mediate osteoclastogenesis and osteoblastogenesis [161-164], although the precise biological mechanisms have not been fully established yet.

Figure 5.1: The structure of vitamin C.



Adapted from Rumsey & Levine (1998) [297].

The formation of the water-soluble vitamin C in plants requires gulonolactone oxidase. The absence of this enzyme in humans means that we cannot synthesise vitamin C ourselves, making it an essential micronutrient that must be consumed as part of the diet [91, 125]. Both active forms of vitamin C, ascorbate and dehydroascorbate **(Figure 5.1)**, are present mainly in plant-based foods such as fruits and vegetables with the highest concentrations found in citrus and soft fruits, their fruit juices and some vegetables including peppers and broccoli [145, 225]. Vitamin C is also available as a supplement in the form of synthetic L-ascorbic acid. It is chemically identical to the natural form, and there are no reported differences in their bioavailability [298].

The mean dietary intake of the vitamin in the UK population is 94.1 mg/d as estimated by the National Diet and Nutrition Survey 2008/2009 for people aged 19-64 years. This is around 235% higher than the UK recommended nutrient intake (RNI) of 40 mg/d. Dietary intake and plasma concentrations of vitamin C when plotted against each other show a sigmoidal

relationship [299, 300]. Average vitamin C intakes (60-100 mg/d) reflect plasma levels of around 40-60 µM/l. Higher intakes result in a progressive flattening of the curve and very high intakes of 400 mg/d and above appear to saturate vitamin C in plasma at concentrations of 70-85 µmol/l [300]. Vitamin C supplements may increase circulating levels more than twofold, although these effects appear to be only short-lived [301]. Vitamin C status is influenced by a number of biological and lifestyle factors including age, sex, BMI, body fat distribution, fat-free mass, smoking and infection [227, 302-306]. For example, women tend to have higher circulating levels of vitamin C than men, possibly due to a volumetric dilution [227], but also due to higher energy-adjusted dietary intakes [224]. Moreover, smokers have lower blood vitamin C levels independent of dietary intake compared to non-smokers [306], and it is thought that this is a result of the higher turnover of vitamin C to counteract the increased level of oxidative stress in smokers [307].

The importance of vitamin C in collagen formation has previously been linked to osteoporosis and fracture prevention, and positive associations between vitamin C intake and BMD have previously been reported [91, 133, 165], although the evidence is limited, especially in men.

5.2.1 The potential role of vitamin C in bone health

5.2.1.1 Osteoclastogenesis

Vitamin C has been suggested to mediate osteoclast differentiation and possibly apoptosis [163, 308] and findings have been relatively consistent. In cell cultures containing both osteoblasts and osteoclasts, vitamin C promoted osteoclastogenesis [309-311] and this was associated with an increase in RANKL expression [310]. In concordance with these findings, vitamin C deficiency resulted in a decrease in osteoclast differentiation [310, 311]. However, in cultures containing only osteoclasts, stimulatory effects [312] as well as inhibitory effects [163, 309, 313] of vitamin C on osteoclast differentiation have been reported. Recent *in vitro* findings have helped explain these contradictory results by showing that vitamin C at a concentration of 50 µg/ml initially exhibited pro-oxidant activity resulting in an increase in the number, size and nucleation of osteoclasts; although vitamin C initiated accelerated osteoclast death at later stages[308]. Deficiency studies are in agreement with most previous findings, indicating that vitamin C deficiency in animal models stimulated osteoclastogenesis via the up-regulation of the RANKL/RANK pathway[161, 163]. Moreover, mice supplemented with vitamin C had a reduction in RANKL expression [161].

5.2.1.2 Osteoblastogenesis

To date, there is consistent experimental evidence for a beneficial role of vitamin C in osteoblastogenesis. For example, a decrease in the number of osteoblasts and suppressed osteoblast differentiation has previously been observed in vitamin C deficient mice [161]. In

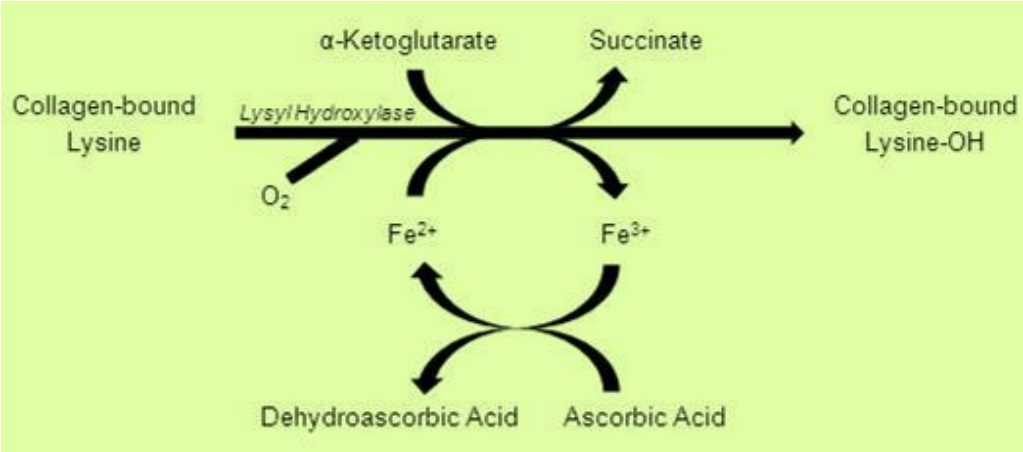
concordance with these findings, an increase in the number of osteoblasts following vitamin C treatment has been reported from *in vitro* work [314]. Furthermore, studies using osteoblast-like cell cultures including human tissue have shown that osteoblast proliferation and differentiation was enhanced with the addition of vitamin C [162, 164, 314, 315]. Concentrations of 50 µg/ml and 200 µg/ml vitamin C have previously been suggested as optimal and maximum concentrations for this effect [162, 164].

Initially, work from the 1990s suggested that vitamin C may be important for osteoblastogenesis through stimulating collagen synthesis [314, 315], although recent evidence suggests the underlying mechanisms are more complex. For example, vitamin C has been reported to mediate gene expression of a number of genes involved in pre-osteoblast cell activities including growth, metabolism, communication and death [316]. Furthermore, studies have shown that the expression of PPAR-γ, an essential transcription factor in adipogenesis [18], may mediate osteoblast differentiation resulting in bone loss [317, 318]. Recently, these findings have been investigated further and a link to vitamin C established. An *in vivo* study reported that PPAR-γ expression in osteoblasts was significantly up-regulated in vitamin C deficient mice and was accompanied by suppressed osteoblast differentiation; whereas treatment with vitamin C mediated PPAR-γ expression to almost normal levels [161].

5.2.1.3 Bone collagen synthesis

Experimental evidence for a role of vitamin C in bone collagen synthesis by osteoblasts is well established. For example, early *in vitro* work reported that collagen synthesis increased more than four fold in the presence of ascorbate [319]. The underlying mechanisms for this are thought to relate to the role of vitamin C in stimulating both the quantity and the quality of collagen synthesis. For the former, vitamin C is an important initiator of collagen synthesis in osteoblasts [136], possibly via stimulating pro-collagen type I mRNA [320, 321]; whereas for the latter, vitamin C is an essential activator of enzymes involved in the hydroxylation of amino acid residues within collagen fibres [7-10, 322]. The hydroxylation reaction, illustrated in **Figure 5.2**, takes place in the endoplasmic reticulum [323] and enables the formation of covalent bonds between the amino acid residues, increasing overall collagen strength [136]. The hydroxylation of collagen lysine requires lysyl hydroxylase in combination with a number of cofactors including oxygen, ferrous ion, a reducing agent such as ascorbic acid and α-ketoglutarate to form collagen hydroxylysine [9, 10]. The importance of vitamin C in this reaction is in the reduction of ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+}) which in turn activates lysyl hydroxylase. Similar to lysine, the hydroxylation of collagen proline requires prolyl hydroxylase and cofactors identical to those mentioned above.

Figure 5.2: The hydroxylation of lysine in collagen fibres.



Adapted from Medeiros & Wildman (2011) [(324)].

Evidence from *in vitro* studies has shown that compounds including the amino acid cysteine and the strong reducing agent dithiothreitol may replace vitamin C in this hydroxylation reaction to a minor extent [(325-327)]. However, vitamin C was shown to be the most effective reducing agent [(322)] and it is currently unknown whether these *in vitro* effects would also be observed in humans. Previous *in vitro* and *in vivo* studies have also indicated that vitamin C deficiency was associated with the formation of underhydroxylated and unhydroxylated collagen [(328, 329)], highlighting the importance if the vitamin in this process.

To date, *in vitro* evidence has shown that vitamin C increases the hydroxylation of amino acid residues [(330)], and a dose-response relationship between vitamin C and type I collagen was recently reported in an *in vitro* study, with the highest amounts of collagen found to be present at vitamin C concentrations of 200 µg/ml compared to 100 µg/ml and 25 µg/ml [(162)]. This suggests that vitamin C deficiency may lead to inadequate hydroxylation of collagen fibres, subsequently mediating bone structure and decreasing the overall strength and stability of bone. As both bone and cartilage contain a structurally stable network of collagen, a reduction in the quantity and quality of collagen resulting from inadequate vitamin C intake may potentially be a risk factor for the development of osteoporosis and associated fractures.

5.2.2 Associations between vitamin C and bone health in previous studies

My recent review of the literature on vitamin C and bone health [(168)] highlighted that, to date, there is evidence from epidemiological studies for a potential role of different forms of vitamin C in reducing osteoporosis and fracture risk, although evidence from RCTs is currently less well defined. In these studies, bone health was investigated as i) fracture incidence as the best indicator of bone health, ii) BMD using DXA as the gold standard method or other measurements including ultrasound, and iii) biochemical markers of bone formation including BSALP, OC, PINP and PICP, as well as markers of bone resorption including PYD, DPD, CTx and NTx. The following

section will review previously published studies which investigated associations between vitamin C and osteoporosis and fracture risk. RCTs as the best indicator of causality will be discussed first, and this will be followed by observational studies in hierarchical order of decreasing ability to determine causality.

5.2.2.1 Intervention studies and randomised controlled trials

A summary table of intervention studies investigating potential associations between vitamin C and bone health can be found in **Appendix 2, Table A2.1**. RCTs are the best studies for inferring causality and for determining which factors influence disease. Thus, they are the gold standard as they limit both selection biases and confounding. To my knowledge, there is only one such published RCT with a double-blind design that has examined the effects of vitamin C supplementation on bone density [331]. The trial compared the effects of taking a placebo *vs.* a combination of vitamin C and E supplements on bone density in three groups of 30 men and women for 12 months. The two intervention groups received 400 IU of vitamin E daily and either 500 mg/d or 1000 mg/d of vitamin C. The group with the highest vitamin C intake had significantly less hip bone loss compared to the placebo group (effect sizes and *P*-values not reported), although no such observations were made at the lumbar spine. However, the study criteria allowed for inclusion of smokers and of participants with controlled chronic disease which may have biased the study outcomes. Moreover, the trial was undertaken in a small number of participants. Furthermore, the trial only investigated the additive effects of the vitamins. Thus, it remains unclear to what extent vitamin C was involved in preventing bone loss.

Two intervention studies used a combination of an exercise programme and supplementation with vitamin C and E [332, 333]. One trial was a randomised placebo-controlled pilot study in 34 women who followed an intervention of 60 minutes of resistance training three times per week and daily supplementation with vitamin C (1000 mg/d) and E (600 mg/d) for six months. They were randomised into four treatment groups of either placebo, vitamins, exercise and placebo, or exercise and vitamins [332]. BMD of the lumbar spine, but not the femoral neck, decreased significantly by 1% in the placebo group over six months (BMD pre: 1.01 ± 0.17 g/cm^2; BMD post: 1.00 ± 0.16 g/cm^2; $P<0.05$) and was maintained in the other groups. No additive effects of the exercise intervention and the vitamin supplementation were found. However, the results may have been biased by changes in dietary habits as a reduction in vitamin C intake over the course of the study period was reported for the vitamin group. Moreover, the study did not report whether the outcome was assessed in a blind fashion. The second study, a two month intervention in 13 men and women, included an hour of aerobic exercise three times per week and the daily use of vitamin C (500 mg/d) and vitamin E (100 mg/d) supplements for all subjects [333]. Although markers of calcium homeostasis improved significantly with parathyroid hormone decreasing and vitamin D increasing (effect sizes not reported), the bone formation marker

BSALP decreased unexpectedly by 14.5% (P-value not reported). One may be critical about the lack of control group in this study as well as its applicability to the general population considering that the intervention was undertaken in only 13 individuals. Moreover, both intervention studies included regular aerobic exercise as well as the use of vitamin E supplements. Thus, the effects of vitamin C *per se* are impossible to differentiate from these interventions. Exercise has previously been shown to stimulate bone formation resulting in higher BMD and a decreased risk of fracture [(79)]; although the effects of vitamin E on the skeleton are currently equivocal [(334)]. Moreover, both studies were of short duration of only two to six months, although changes in BMD are more likely to be observed after a longer duration of treatment as the rate of bone loss is estimated to be only about 1% per year.

In summary, evidence from previous trials investigating the potential preventative effects of vitamin C in osteoporosis remains equivocal. There are limitations regarding study design, inclusion and exclusion criteria, limited duration of treatment and small sample sizes. Moreover, published intervention studies have used vitamin supplements containing vitamin E in addition to vitamin C and have included exercise programmes during treatment. Future trials should consider having more participants, stricter inclusion and exclusion criteria and interventions consisting of vitamin C supplementation only.

5.2.2.2 Prospective studies

Prospective cohort studies may be used to investigate the aetiology of a disease as the exposure is measured prior to the condition occurring, making data less prone to recall bias than case-control studies. Furthermore, as cases and controls are drawn from the same population, there is less selection bias. To date, only three prospective studies have investigated potential vitamin C to bone associations, and a summary table of these studies can be found in **Appendix 2, Table A2.2**. One study of 944 British men and women (mean age = 72 years) reported that those with higher dietary intakes of vitamin C (99-363 mg/d) compared with lower intakes (7-57 mg/d) lost significantly less BMD at the hip after 2-5 years of follow-up [(166)]. Another study in a US cohort of 606 subjects aged 75 years on average reported that lumbar spine and trochanter BMD loss, but not femoral neck and radial shaft BMD loss, decreased significantly across tertiles of dietary vitamin C intake in men but not in women [(230)]. However, the findings were not consistent across these two studies with results varying mainly for sex and bone site. Potential explanations for this might be differences in measures of dietary intake, bone density measures and adjustment for confounding factors. For example, the first study used 7-day food diaries and did not adjust for important confounders including age, sex and smoking [(166)]. In contrast, the second study used a semi-quantitative FFQ and measured BMD via two different types of bone scans (i.e. DPA at baseline and DXA at follow-up) [(230)]. However, DXA scans have been shown to produce lower

results than DPA scans [335], hence the effect size in this study may be more modest than the true result.

A potential role for vitamin C in fracture prevention has only been investigated in one previous prospective study. The study used a sample of 918 US men and women with a mean age of 75 years and found a risk reduction in hip fracture of 44% for supplemental vitamin C intake (mean: 260 mg/d compared to 0 mg/d) and of 69% for total (dietary and supplemental) vitamin C intake (mean: 313 mg/d compared to 94 mg/d) after 15-17 years of follow-up (RR and 95%CI not reported) [167]. However, no associations between dietary vitamin C intake and the risk of fracture at different sites were reported. Further large prospective cohort studies are needed of older men and women with long follow-up, which investigate fractures as the clinical endpoint of osteoporosis. Furthermore, there is only limited prospective data on a potential link between vitamin C status and bone health. To my knowledge, only one study has investigated plasma vitamin C concentrations and reported no significant associations with changes in hip BMD [166]. Similar issues regarding the adjustment for important confounding factors as discussed above also apply to these prospective studies; and more studies are needed as current evidence is limited.

In conclusion, there is only limited data from three prospective and longitudinal studies investigating potential associations between vitamin C and bone health. Currently, it is difficult to assess the strength of the associations as not all studies reported effect sizes. A greater number of prospective and longitudinal studies and adjustment for confounding factors may help establish more consistent findings of the relationship between vitamin C intake and status with osteoporosis and associated fractures.

5.2.2.3 Case-control studies

Case-control studies, summarised in a table in **Appendix 2, Table A2.3**, are used to examine specific exposures as potential risk factors of a disease in people with and without the condition. The exposures preceded the disease outcome, and thus case-control studies are prone to recall bias where case subjects tend to have a better recollection of specific exposures than the controls. Moreover, selection bias is an issue as both the exposure and the disease outcome are pre-defined. To date, three case-control studies have consistently shown that osteoporosis and fracture patients had lower serum vitamin C concentrations (cases: 17-37 µmol/l; controls: 23-54 µmol/l) and lower plasma vitamin C concentrations (cases: 30 µmol/l; controls: 55 µmol/l) than controls [336-338]. Only one study reported differently, but the authors inferred that their findings reflected the daily consumption of orange juice given at breakfast during the recent hospital admission of their study population, thus reflecting recent rather than habitual food intake [339].

In contrast to vitamin C status measures, findings for potential differences in dietary vitamin C intakes between cases and controls are less consistent. Differences in measures of

dietary intake and relatively small sample sizes may explain some of these inconsistent findings. Although previous case-control studies have reported no significant differences for mean vitamin C intakes [336, 339], associations with the relative risk of osteoporosis and fractures were reported when stratified by quartiles of the populations' intakes although often in a non-linear fashion. For example, Martinez-Ramirez *et al.* (2007) showed a marginally significant fracture risk reduction for participants in the second quartile of vitamin C intake compared to the first (OR = 0.39; 95%CI 0.15-1.00; vitamin C intake range: 204-247 mg/d compared to ≤203 mg/d) [336]. This was not significant for higher vitamin C intakes, possibly due to the high vitamin C intake (mean: 200 mg/d) of the study population. Moreover, Park *et al.* (2011) reported that those in the third quartile of vitamin C intake had a significantly reduced risk of osteoporosis compared to those in the lowest quartile (OR = 0.29; 95%CI 0.09-0.96; vitamin C intake range: 137-176 mg/d compared to ≤92 mg/d) [340].

In conclusion, published case-control studies have shown consistent results for associations between lower vitamin C status in osteoporosis and fracture patients, however findings for dietary vitamin C intakes were less consistent. Moreover, although reported effect sizes appear to be large, this data is limited to only two case-control studies and a dose-response relationship was not apparent in these studies. More case-control studies are needed to help clarify the discrepancies in vitamin C intake between osteoporosis and fracture patients and controls currently reported in the literature.

5.2.2.4 Cross-sectional studies

Cross-sectional studies are used to report the prevalence of a disease in a defined population at a specific point in time. Whether the exposure predated the disease or not cannot be determined. Previous cross-sectional studies investigating vitamin C and bone health associations are summarised in **Appendix 2, Table A2.4**. They have reported inconsistent findings with outcomes related to age [341], menopausal status [169], smoking behaviour [230] and oestrogen use [341]. Nevertheless, positive associations between vitamin C and BMD have previously been reported. For example, higher intakes of dietary vitamin C were associated with 3-5% higher BMD [133]. Moreover, every 100 mg/d increment in vitamin C intake was associated with 0.01-0.02 g/cm^2 higher BMD [91, 169], although there is currently limited understanding of this clinical relevance. In one study, users of vitamin C supplements (mean [range] = 745 mg/d [70-5000 mg/d]) had 4% higher BMD compared to non-users [165]. Moreover, in the same study, users of supplement doses of ≥1000 mg/d had 14% higher BMD than non-users. Although positive associations between dietary and supplemental vitamin C intake and bone density have previously been reported, findings have been inconsistent [124, 131, 230, 342-344], possibly, at least in part, due to differences in the adjustment for confounding factors. The use of different dietary assessment methods as means of measuring dietary vitamin C intake may also explain some of

these discrepancies. Methods have included semi-quantitative FFQs with 97–126 food items [91, 124, 133, 229, 230, 341, 342, 345], three to seven-day dietary records [131, 345] and 24-hour recalls [169, 344]. Total vitamin C intake has not been linked with BMD in women [342, 344, 345]; and both positive and negative associations have been reported in men [230], although the latter findings may have been biased by the population's smoking behaviour. Dietary intakes of vitamin C have previously been shown to be significantly lower in smokers than non-smokers [306] and serum vitamin C levels are lower in smokers independent of dietary vitamin C intake [306, 346]. The exclusion of smokers to the study may have led to more consistent findings.

Potential associations between vitamin C and fracture risk have only been examined in one cross-sectional study which did not find a link between higher dietary vitamin C intakes and self-reported fractures in women [169]. In the same study, men with mean dietary vitamin C intakes of 200 mg/d reported fewer fractures than men with higher or lower intakes. The study also found no significant associations between serum vitamin C concentrations and self-reported fracture risk. One may be critical about the large age range of the study population. As osteoporosis and associated fractures are known to be more prevalent in the elderly population [22], the inclusion of very young participants may explain the non-significant findings.

Cross-sectional data on vitamin C and markers of bone homeostasis is sparse with only two studies investigating potential associations. New *et al.* (2000) found that higher intakes of vitamin C were associated with lower excretion of deoxypyridinoline *(no effect size shown)*, indicating reduced bone resorption [124]. Similarly, Pasco *et al.* (2006) reported a significant association between the duration of vitamin C supplement use and markers of bone resorption, with serum CTx concentrations being 0.022 pg/mL lower for every 1-year supplement use increment [343].

Although there is data from a number of cross-sectional studies investigating vitamin C and bone health relationships, current evidence is not consistent. Effect sizes of present cross-sectional studies are comparable to those previously reported for other dietary factors including potassium, although many studies did not report effect sizes. Future cross-sectional studies investigating the relationship between vitamin C intake and status with osteoporosis and related fractures are needed to address the discrepancies between current cross-sectional studies.

5.2.2.5 Summary of previously published studies

My recent review of the literature on vitamin C and bone health [168] highlighted that only a very few interventions have examined the effects of vitamin C supplementation on changes in BMD and bone turnover markers, and those have reported beneficial effects. However, all previous interventions had mixed treatments using a combination of different vitamins and an exercise intervention, thus making it difficult to disentangle the relative contribution of vitamin C to the shown beneficial effects. Issues regarding study design and duration of treatment were

identified with only one study following the design of a double-blind RCT and all studies having relatively short interventions of 2-12 months. Future RCTs of longer duration are needed that are designed to establish the effects of vitamin C supplementation on different aspects of bone health. My review [168] also highlighted that support for mechanistic studies for a potential link between vitamin C and osteoporosis prevention has come from a variety of epidemiological studies, although differences in study populations, dietary assessment methods, outcome measures and use of confounding factors in statistical analyses may have resulted in inconsistent findings. Epidemiological data from British populations is scarce and data are particularly limited for men. Moreover, the majority of these studies have used FFQs to estimate habitual vitamin C intake, despite the availability of more accurate dietary assessments including food diaries. Published studies have also focused on the intake of vitamin C, although biological markers of nutrient status may be less subjective to factors such as storage, processing and bioavailability [170]. With regards to bone health as the health outcome, data are particularly lacking for fracture risk. Furthermore, some studies stratified by additional factors such as smoking status, oestrogen use or calcium intake, hence reducing the applicability of their findings to greater populations. More epidemiological studies in the general population are needed to address some of these limitations.

5.2.3 Chapter aims and objectives

In order to address some of these limitations, this chapter aimed to:

i) Investigate the cross-sectional associations between vitamin C intake from diet and from the combined total intake of diet and supplements, which is available from a carefully constructed ViMiS database established by EPIC-Norfolk [213], as well as plasma vitamin C concentrations with the heel ultrasound parameters BUA and VOS.

ii) Examine the prospective associations between dietary and total vitamin C intake and plasma vitamin C with the risk of fracture at the hip, spine and wrist in a British population of men and women aged between 39 and 79 years at baseline.

iii) Undertake a number of sensitivity analyses for both of these aims by a) using residuals of vitamin C intake [347], b) excluding current vitamin C supplement users, c) investigating vitamin C intake per kilogram bodyweight, and d) restricting the cohort to older men and women aged 65 years and over.

The findings will provide more evidence in a British population, particularly in men, where data is limited; and more evidence for potential vitamin C to bone associations using more accurate dietary information (7dDD and ViMiS database) compared to most previous studies (FFQs). Moreover, this study will provide novel findings of potential associations between vitamin C

status and fracture risk. It was hypothesised that vitamin C intake and status are positively associated with measures of bone density and inversely associated with the risk of fracture.

5.3 Methods

As discussed in Chapter 2 (page 40), a cross-sectional study and a prospective case-cohort study were performed on a representative sample of men and women of the EPIC-Norfolk cohort study. For both types of studies, analyses using vitamin C as the predictor variable were performed using quintiles and were undertaken for i) intake from the diet, ii) total intake which accounted for intake from both diet and supplements, and iii) plasma concentrations. The first study was undertaken in a random sub-cohort of 4000 EPIC-Norfolk participants and used multiple regression with multivariate adjustment to assess the cross-sectional relation of quintiles of vitamin C to BUA and VOS. Both BUA and VOS are measures of heel ultrasound, but BUA is an indicator of the structural organisation of bone, whereas VOS determines bone stiffness [(63)]. In the second study, undertaken in a case-cohort sub-sample (*n*=5319) of the EPIC-Norfolk cohort, Kaplan-Meier survival curves alongside log-rank tests of equality were computed to evaluate differences in crude total osteoporotic fracture incidence over the median 12.6-year follow-up between the quintile groups. Then, Prentice-weighted Cox proportional hazard ratios [(221)] were used to investigate the prospective relations of quintiles of vitamin C to fracture risk for three important fracture sites (hip, spine and wrist). In both studies, potential associations between the top two quintiles referent to the lowest quintile of vitamin C intake or plasma concentrations were investigated, as a number of previous studies reported a bell-shaped dose-response of the nutrient [(133, 169)]. As previously discussed, all analyses were stratified by sex and adjusted for relevant confounders using an unadjusted and two multivariate models (Chapter 2, page 49). The final model included age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. Moreover, the procedures for dealing with missing data and the number of exclusions in each study are discussed in detail in Chapter 3 (pages 55-57).

Following the primary analysis, further explorative investigations were undertaken for both the cross-sectional study of heel ultrasound and the prospective study of fracture risk. Despite adding total energy intake to the multivariate model, dietary and total vitamin C intakes were also adjusted for energy using the residual method of Willett because this is a useful way to identify the actual variation in vitamin C intake whilst energy intake is being held constant [(347)]. For this, dietary and total vitamin C intakes were regressed on total energy intake. The calculated vitamin C residual, which is uncorrelated with energy intake, was then regressed on BUA and VOS as well as total osteoporotic fracture risk using the full covariate model.

In order to evaluate potential associations between vitamin C from the diet independent of supplement use, a sensitivity analysis was also undertaken. This is because supplement users compared to con-users tend to have higher dietary intakes of most micronutrients [262, 348, 349]. Hence, subjects, who had reported the current use of vitamin C supplements, were excluded from the subsequent regression analyses of the final covariate model.

A number of studies have reported that the distribution of vitamin C in plasma and tissues may be influenced by body weight [302]. Thus, for each participant, dietary and total intakes and plasma concentrations of vitamin C were also calculated per kilogram bodyweight, before being regressed on BUA, VOS and total fracture risk using the full covariate model.

Previous studies have indicated that fracture rates increase progressively with increasing age [22], possibly because osteoporosis is unlikely to have progressed to its clinical endpoint at a young age. Preliminary analyses presented in Chapter 3 (page 68) showed that this was applicable to the present study population. Thus, associations between vitamin C and total osteoporotic fracture risk adjusted using the full covariate model were also determined in a smaller case-cohort sample of older men and women aged 65-79 years at baseline.

5.4 Results

5.4.1 Descriptive statistics stratified by quintiles of dietary vitamin C intake

Characteristics of the 4711 EPIC-Norfolk men and women stratified by quintiles of dietary vitamin C intake are presented in **Table 5.1**. In the 1957 men, mean±SD dietary vitamin C intakes for the quintiles were: Q1 33.9±8.8 mg/d, Q2 53.3±5.0 mg/d, Q3 73.1±6.7 mg/d, Q4 101.1±9.4 mg/d and Q5 166.8±50.1 mg/d. In the 2754 women, mean±SD dietary vitamin C intakes for each quintile were similar: Q1 35.6±9.3 mg/d, Q2 57.8±5.5 mg/d, Q3 78.5±6.8 mg/d, Q4 105.9±9.6 mg/d and Q5 168.2±42.2 mg/d. There were no significant differences in age, family history of osteoporosis and the use of steroids between the quintile groups in both sexes. However, with higher dietary vitamin C intakes, the use of vitamin D supplements increased, whereas the prevalence of current smoking decreased (all $P<0.001$). In women, BMI differed significantly between the quintile groups ($P=0.010$). Moreover, women with higher dietary vitamin C intakes were more physically active and more likely to use calcium supplements than women with lower intakes ($P\leq0.007$). BMI, physical activity and the use of calcium supplements did not differ significantly between quintiles of dietary vitamin C intake in men. There were no differences in menopausal status and HRT between the quintile groups in women. As expected, mean plasma vitamin C concentrations increased significantly with higher dietary intakes of vitamin C in both sexes ($P<0.001$).

5.4.2 Descriptive statistics stratified by quintiles of plasma vitamin C

Characteristics of the 4130 men and women stratified by quintiles of plasma vitamin C are shown in **Table 5.2**. In the 1754 men, mean±SD plasma vitamin C concentrations for the quintiles were: Q1 20.2±6.5 µmol/l, Q2 37.7±3.8 µmol/l, Q3 48.0±2.5 µmol/l, Q4 56.5±2.4 µmol/l and Q5 71.3±10.0 µmol/l. In the 2376 women, plasma vitamin C intakes for each quintile were: Q1 30.3±9.9 µmol/l, Q2 50.1±3.3 µmol/l, Q3 59.5±2.3 µmol/l, Q4 68.2±2.9 µmol/l and Q5 85.8±13.4 µmol/l. Men and women with higher plasma vitamin C levels were significantly younger, lighter, less likely to smoke and more physically active than those with lower plasma levels ($P \leq 0.013$). Women with higher *vs.* lower plasma levels were also less likely to be postmenopausal ($P=0.018$), but there were no differences in HRT between the groups. There were no significant differences in family history of osteoporosis and the use of steroids between the quintile groups in both sexes, but the use of calcium and vitamin D supplements increased significantly across quintiles of plasma vitamin C levels ($P<0.001$). As expected, mean dietary vitamin C intake increased significantly with higher plasma concentrations in both sexes ($P<0.001$).

Table 5.1: Baseline characteristics of the 1957 men and 2754 women of the EPIC-Norfolk case-cohort by quintiles of dietary vitamin C intake.

	Men											Women										
Vitamin C intake (mg/d)	**Quintile 1 0 – 45.2**		**Quintile 2 45.3 – 61.9**		**Quintile 3 62.0 – 85.6**		**Quintile 4 85.7 – 119.6**		**Quintile 5 119.7 - 471.4**		**P-trend**	**Quintile 1 0.1 – 48.4**		**Quintile 2 48.5 – 66.9**		**Quintile 3 67.0 – 90.5**		**Quintile 4 90.6 – 124.2**		**Quintile 5 124.3 – 405.3**		**P-trend**
	n = 392		***n = 391***		***n = 392***		***n = 391***		***n = 391***			***n = 551***		***n = 551***		***n = 551***		***n = 551***		***n = 550***		
Mean (SD)																						
Age (years)	59.4	(10.0)	59.7	(9.7)	59.7	(9.1)	60.4	(9.5)	59.4	(9.6)	*P*=0.64	60.2	(10.2)	59.3	(9.5)	60.0	(9.6)	59.8	(9.3)	59.7	(9.1)	*P*=0.69
BMI (kg/m^2)	26.6	(3.4)	26.4	(3.2)	26.6	(3.4)	26.5	(3.4)	26.4	(3.2)	*P*=0.63	26.4	(4.4)	26.3	(4.5)	26.2	(4.4)	26.0	(4.0)	25.9	(4.3)	*P*=0.023
Plasma vitamin C (μmol/l)†	34.2	(17.2)	41.4	(17.7)	45.4	(16.6)	51.3	(14.7)	59.1	(14.0)	*P*<0.001	44.9	(20.5)	55.5	(20.4)	58.6	(17.2)	63.1	(16.2)	68.8	(17.5)	*P*<0.001
n (%)																						
Menopausal Status																						*P*=0.98
Pre-mp	-	-	-	-	-	-	-	-	-	-		86	(15.6)	87	(15.8)	84	(15.3)	82	(14.9)	74	(13.5)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		21	(3.8)	24	(4.4)	23	(4.2)	28	(5.1)	31	(5.6)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		90	(16.3)	89	(16.2)	88	(16.0)	89	(16.2)	92	(16.7)	
Post-mp	-	-	-	-	-	-	-	-	-	-		354	(64.3)	351	(63.7)	356	(64.6)	352	(63.9)	353	(64.2)	
HRT																						*P*=0.86
Current User	-	-	-	-	-	-	-	-	-	-		92	(16.7)	99	(18.0)	97	(17.6)	83	(15.1)	101	(18.4)	
Former User	-	-	-	-	-	-	-	-	-	-		64	(11.6)	61	(11.1)	69	(12.5)	62	(11.3)	68	(12.4)	
Never Used	-	-	-	-	-	-	-	-	-	-		395	(71.7)	391	(71.0)	385	(69.9)	406	(73.7)	381	(69.3)	
Smoking											*P*<0.001											*P*<0.001
Current smoker	88	(22.5)	51	(13.0)	43	(11.0)	34	(8.7)	22	(5.6)		125	(22.7)	59	(10.7)	69	(12.5)	49	(8.9)	41	(7.5)	
Former smoker	202	(51.5)	241	(61.6)	222	(56.6)	194	(49.6)	222	(56.8)		171	(31.0)	186	(33.8)	189	(34.3)	170	(30.9)	174	(31.6)	
Never smoked	102	(26.0)	99	(25.3)	127	(32.4)	163	(41.7)	147	(37.6)		255	(46.3)	306	(55.5)	293	(53.2)	332	(60.3)	335	(60.9)	
Physical activity											*P*=0.21											*P*<0.001
Inactive	141	(36.0)	112	(28.6)	111	(28.3)	129	(33.0)	121	(31.0)		224	(40.7)	195	(35.4)	183	(33.2)	156	(28.3)	149	(27.1)	
Mod. inactive	88	(22.5)	95	(24.3)	108	(27.6)	88	(22.5)	92	(23.5)		168	(30.5)	181	(32.9)	164	(29.8)	194	(35.2)	170	(30.9)	
Mod. active	87	(22.2)	92	(23.5)	93	(23.7)	73	(18.7)	91	(23.3)		95	(17.2)	102	(18.5)	127	(23.1)	118	(21.4)	135	(24.6)	
Active	76	(19.4)	92	(23.5)	80	(20.4)	101	(25.8)	87	(22.3)		64	(11.6)	73	(13.3)	7	(14.0)	83	(15.1)	96	(17.5)	
Family history of OP	9	(2.3)	12	(3.1)	7	(1.8)	16	(4.1)	14	(3.6)	*P*=0.31	28	(5.1)	35	(6.4)	31	(5.6)	32	(5.8)	28	(5.1)	*P*=0.88
Steroids	13	(3.3)	18	(4.6)	14	(3.6)	11	(2.8)	12	(3.1)	*P*=0.69	28	(5.1)	22	(4.0)	18	(3.3)	24	(4.4)	22	(4.0)	*P*=0.66
Calcium supp.	4	(1.0)	2	(0.5)	6	(1.5)	7	(1.8)	6	(1.5)	*P*=0.52	18	(3.3)	30	(5.4)	34	(6.2)	24	(4.4)	49	(8.9)	*P*=0.001
Vitamin D supp.	50	(12.8)	74	(18.9)	107	(27.3)	97	(24.8)	102	(26.1)	*P*<0.001	131	(23.8)	168	(30.5)	171	(31.0)	191	(34.7)	214	(38.9)	*P*<0.001

Values are means (standard deviations) or numbers (frequencies). Abbreviations: Mp, menopausal; Family history of OP, family history of osteoporosis; Supp., supplements.
† Plasma vitamin C levels were available for 1754 men and 2376 women.

Table 5.2: Baseline characteristics of the 1754 men and 2376 women of the EPIC-Norfolk case-cohort stratified by quintiles of plasma vitamin C.

	Men											Women										
Plasma vitamin C levels (µmol/l)	**Quintile 1 3 – 30**		**Quintile 2 31 – 43**		**Quintile 3 44 – 52**		**Quintile 4 53 – 61**		**Quintile 5 62 – 132**		**P-trend**	**Quintile 1 4 – 43**		**Quintile 2 44 – 55**		**Quintile 3 56 – 63**		**Quintile 4 64 – 73**		**Quintile 5 74 – 170**		**P-trend**
	n = 355		***n = 373***		***n = 364***		***n = 317***		***n = 345***			***n = 491***		***n = 505***		***n = 437***		***n = 488***		***n = 455***		
Mean (SD)																						
Age (years)	61.8	(9.6)	60.2	(9.7)	59.3	(9.1)	57.9	(9.3)	58.6	(9.8)	*P*<0.001	61.1	(9.4)	59.4	(10.0)	59.0	(9.7)	59.5	(9.6)	59.4	(8.8)	*P*=0.013
BMI (kg/m^2)	26.6	(3.4)	27.0	(3.4)	26.7	(3.1)	26.3	(3.0)	25.5	(2.9)	*P*<0.001	27.0	(4.7)	26.8	(4.6)	25.7	(4.1)	25.5	(3.8)	25.3	(3.8)	*P*<0.001
Vitamin C intake (mg/d)	51.9	(23.4)	72.3	(35.7)	88.6	(47.5)	99.9	(48.1)	119.8	(67.6)	*P*<0.001	60.5	(34.0)	83.4	(46.7)	92.6	(45.5)	102.6	(49.7)	112.7	(55.2)	*P*<0.001
n (%)																						
Menopausal Status																						*P*=0.018
Pre-mp	-	-	-	-	-	-	-	-	-	-		50	(10.2)	93	(18.4)	78	(17.8)	74	(15.2)	69	(15.2)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		18	(3.7)	27	(5.3)	25	(5.7)	17	(3.5)	17	(3.7)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		86	(17.5)	70	(13.9)	68	(15.6)	89	(18.2)	73	(16.0)	
Post-mp	-	-	-	-	-	-	-	-	-	-		337	(68.6)	315	(62.4)	266	(60.9)	308	(63.1)	296	(65.1)	
HRT																						*P*=0.74
Current User	-	-	-	-	-	-	-	-	-	-		88	(17.9)	84	(16.6)	76	(17.4)	82	(16.8)	89	(19.6)	
Former User	-	-	-	-	-	-	-	-	-	-		52	(10.6)	55	(10.9)	54	(12.4)	53	(10.9)	61	(13.4)	
Never Used	-	-	-	-	-	-	-	-	-	-		351	(71.5)	366	(72.5)	307	(70.2)	353	(72.3)	305	(67.0)	
Smoking											*P*<0.001											*P*<0.001
Current smoker	90	(25.3)	39	(10.5)	25	(6.9)	19	(6.0)	25	(7.2)		103	(21.0)	61	(12.1)	42	(9.6)	42	(8.6)	32	(7.0)	
Former smoker	188	(53.0)	222	(59.5)	201	(55.2)	171	(53.9)	188	(54.5)		154	(31.4)	144	(28.5)	143	(32.7)	172	(35.3)	157	(34.5)	
Never smoked	77	(21.7)	112	(30.0)	138	(37.9)	127	(40.1)	132	(38.3)		234	(47.6)	300	(59.4)	252	(57.7)	274	(56.1)	266	(58.5)	
Physical activity											*P*=0.006											*P*=0.004
Inactive	135	(38.0)	112	(30.0)	121	(33.2)	88	(27.8)	83	(24.1)		197	(40.1)	157	(31.1)	140	(32.0)	146	(29.9)	122	(26.8)	
Mod. inactive	83	(23.4)	96	(25.7)	93	(25.6)	72	(22.7)	85	(24.6)		146	(29.7)	179	(35.4)	145	(33.2)	156	(32.0)	152	(33.4)	
Mod. active	65	(18.3)	95	(25.5)	72	(19.8)	74	(23.3)	88	(25.5)		96	(19.6)	98	(19.4)	85	(19.5)	103	(21.1)	110	(24.2)	
Active	72	(20.3)	70	(18.8)	78	(21.4)	83	(26.2)	89	(25.8)		52	(10.6)	71	(14.1)	67	(15.3)	83	(17.0)	71	(15.6)	
Family history of OP	6	(1.7)	13	(3.5)	9	(2.5)	11	(3.5)	11	(3.2)	*P*=0.55	27	(5.5)	29	(5.7)	16	(3.7)	34	(7.0)	29	(6.4)	*P*=0.26
Steroids	16	(4.5)	13	(3.5)	15	(4.1)	9	(2.8)	8	(2.8)	*P*=0.50	28	(5.7)	27	(5.4)	17	(3.9)	15	(3.1)	12	(2.6)	*P*=0.06
Calcium supp.	0	(0.0)	3	(0.8)	1	(0.3)	7	(2.2)	13	(3.8)	*P*<0.001	11	(2.2)	18	(3.6)	24	(5.5)	39	(8.0)	45	(9.9)	*P*<0.001
Vitamin D supp.	40	(11.3)	79	(21.2)	72	(19.8)	81	(25.6)	113	(32.8)	*P*<0.001	103	(21.0)	138	(27.3)	149	(34.1)	184	(37.7)	198	(43.5)	*P*<0.001

Values are means (standard deviations) or numbers (frequencies). Abbreviations: Mp, menopausal; Family history of OP, family history of osteoporosis; Supp., supplements.

5.4.3 Associations between vitamin C and heel ultrasound

Associations between the two bone density parameters BUA (in dB/MHz) and VOS (in m/s) with dietary and total intakes and plasma levels of vitamin C are presented in **Figure 5.3** for men and in **Figure 5.4** for women. The results are discussed in detail below. Briefly, we found that dietary and total intakes of vitamin C were significantly positively associated with VOS in men and with BUA in women.

5.4.3.1 Primary analysis

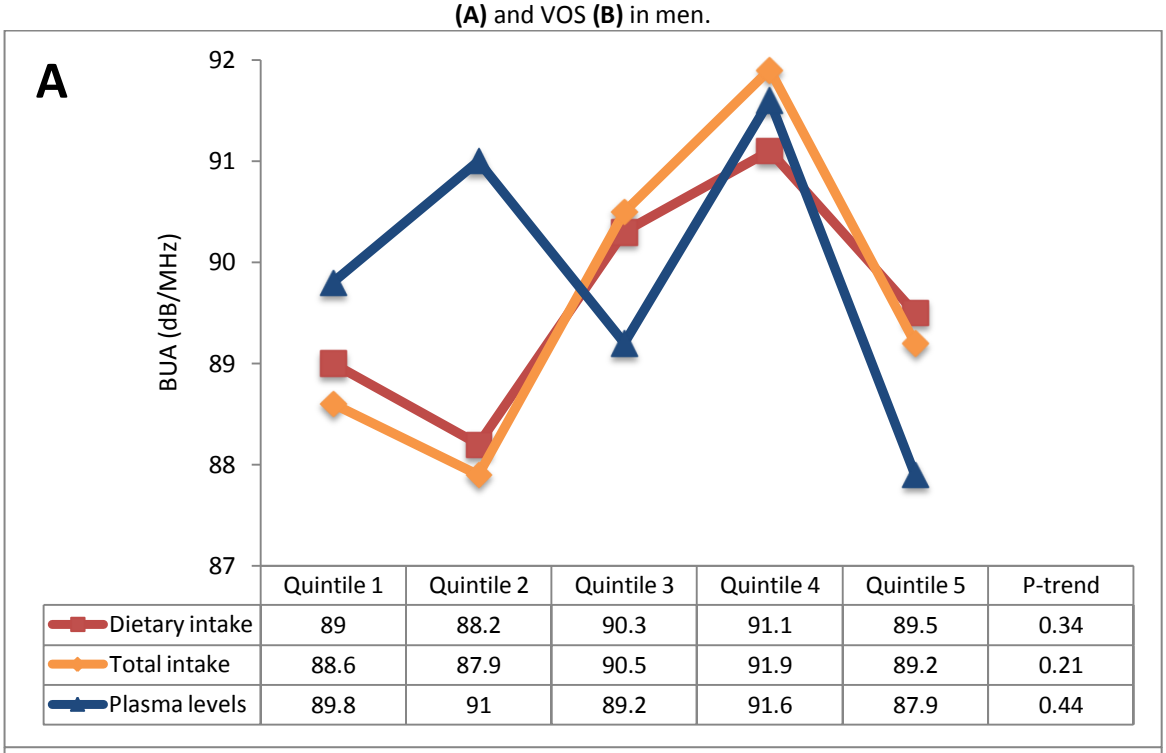
Dietary and total vitamin C intake and heel ultrasound

In univariate analyses, dietary and total vitamin C intake did not correlate with heel ultrasound in men, but a small yet significant correlation was found between dietary intake and BUA in women (r=0.06, *P*<0.05).

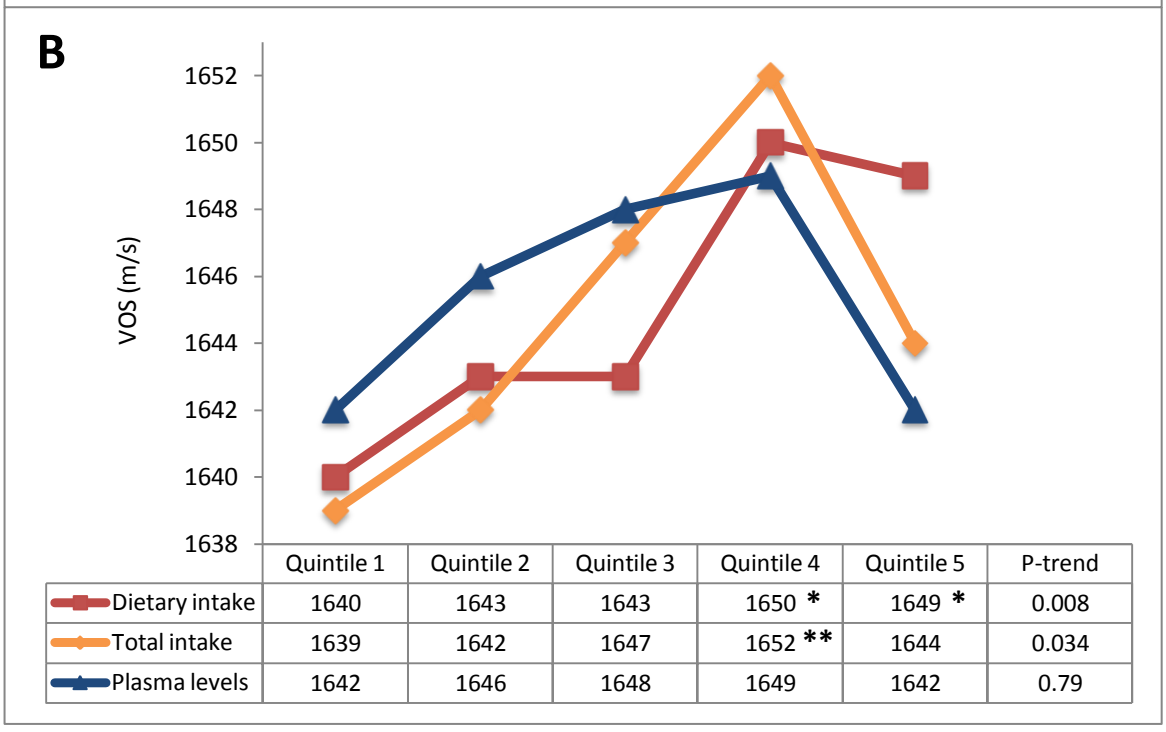
In the 967 men, there was a positive linear relationship between VOS and quintiles of dietary vitamin C intake, and this association remained significant even after adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids, energy intake, dietary calcium intake and the use of calcium and vitamin D supplements (β 2.47 m/s per quintile, *P*-trend=0.008; **Figure 5.3B**). Moreover, there were significant differences in VOS between the two upper quintiles of dietary vitamin C intake referent to the lowest quintile of intake, with VOS being 0.6% higher for quintile 4 (β 9.65 m/s, *P*=0.019) and 0.5% higher for quintile 5 (β 8.79 m/s, *P*=0.035). Results were similar when investigating total vitamin C intake estimated from both diet and supplements. There was a significant linear association between total intake and VOS (β 2.00 m/s per quintile; *P*-trend=0.034), VOS was 0.8% higher in quintile 4 compared to quintile 1 (β 12.82 m/s; *P*=0.002). BUA was not found to be associated with neither dietary nor total vitamin C intake after multivariate adjustment in men **(Figure 5.3A)**.

In contrast to men, there was no relation between VOS and dietary and total vitamin C intake in the 1356 women **(Figure 5.4B)**. However, BUA increased significantly across quintiles of dietary vitamin C intake in a linear fashion after adjustment for important confounding factors (β 0.81 dB/MHz per quintile, *P*-trend=0.004; **Figure 5.4A**). There were also significant and positive differences of 3.7% and 5.8% between quintiles 4 and 5 of dietary vitamin C intake referent to quintile 1, respectively (β 2.56 dB/MHz, *P*=0.041 and β 4.06 dB/MHz, *P*=0.001). In women, total vitamin C intake showed very similar associations with heel ultrasound, where the trend across quintiles was linear for BUA (β 0.71 dB/MHz per quintile, *P*-trend=0.014) and not significant for VOS.

Figure 5.3: Associations between dietary intakes and plasma concentrations of vitamin C with mean BUA **(A)** and VOS **(B)** in men.



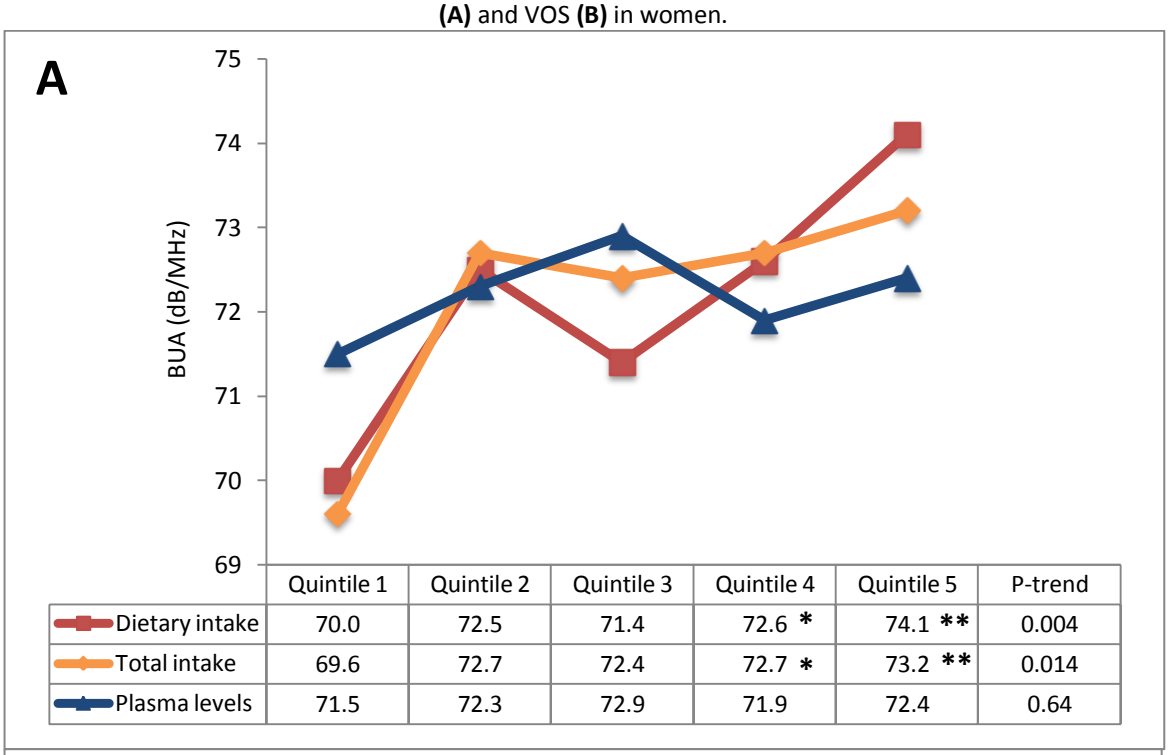
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	89	88.2	90.3	91.1	89.5	0.34
Total intake	88.6	87.9	90.5	91.9	89.2	0.21
Plasma levels	89.8	91	89.2	91.6	87.9	0.44



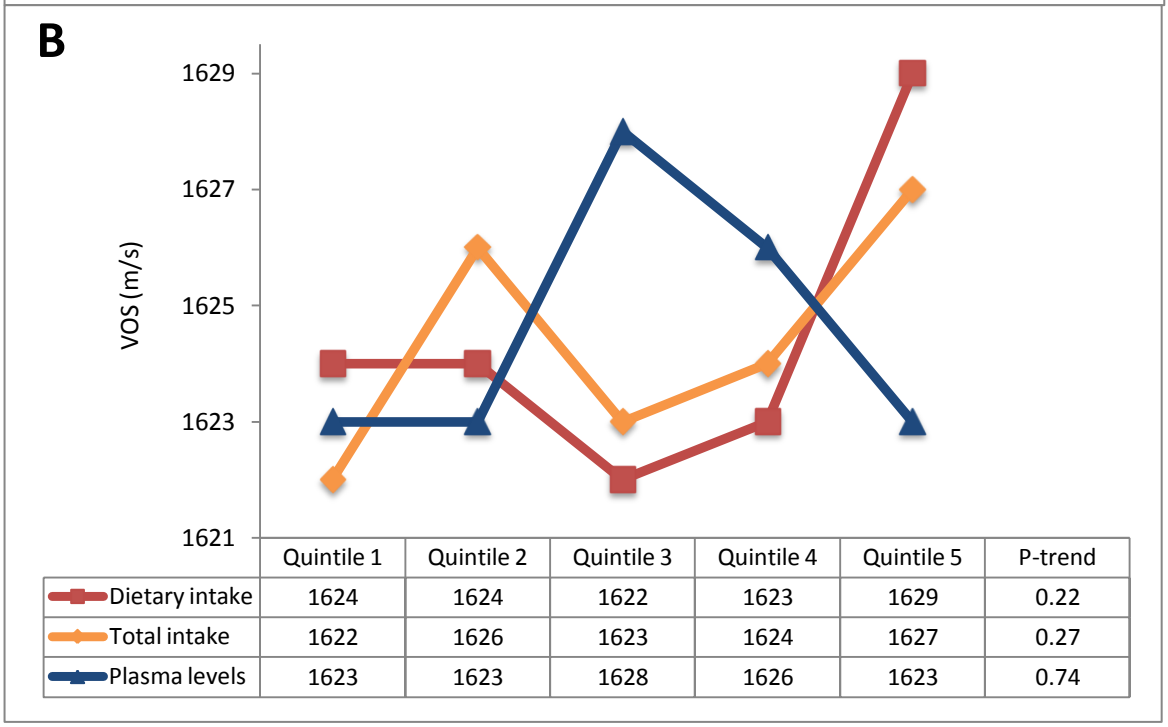
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	1640	1643	1643	1650 *	1649 *	0.008
Total intake	1639	1642	1647	1652 **	1644	0.034
Plasma levels	1642	1646	1648	1649	1642	0.79

*Mean vitamin C intake or plasma concentrations for quintile 1 and 5 ranged from 37-173 mg/d for dietary intake, 38-240 mg/d for total intake and 24-72 µmol/l for plasma levels. Standard error of the mean (SE) was 1.2-1.4 dB/MHz for BUA and 2.8-3.1 m/s for VOS. Total intake is the sum of vitamin C intake from the diet and from supplements. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05 and **P<0.01. n=967 for intake and n=884 for plasma concentrations.*

Figure 5.4: Associations between dietary intakes and plasma concentrations of vitamin C with mean BUA **(A)** and VOS **(B)** in women.



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	70.0	72.5	71.4	72.6 *	74.1 **	0.004
Total intake	69.6	72.7	72.4	72.7 *	73.2 **	0.014
Plasma levels	71.5	72.3	72.9	71.9	72.4	0.64



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	1624	1624	1622	1623	1629	0.22
Total intake	1622	1626	1623	1624	1627	0.27
Plasma levels	1623	1623	1628	1626	1623	0.74

*Mean vitamin C intake or plasma concentrations for quintile 1 and 5 ranged from 40-172 mg/d for dietary intake, 42-361 mg/d for total intake and 34-88 µmol/l for plasma levels. Standard error of the mean (SE) was 0.9-1.0 dB/MHz for BUA and 2.2-2.4 m/s for VOS. Total intake is the sum of vitamin C intake from the diet and from supplements. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05 and **P<0.01. n=1356 for intake and n=1193 for plasma concentrations.*

Plasma vitamin C concentrations and heel ultrasound

In univariate analyses, plasma vitamin C concentrations did not correlate with heel ultrasound in participants of the EPIC-Norfolk cohort.

Figures 5.3-5.4 also show the relationship between quintiles of plasma vitamin C and heel bone density in men and women, respectively. The categorisation of subjects into sex-specific quintiles of plasma vitamin C concentrations differed between the two sexes. The plasma concentration range of quintile 1 in women (4-46 µmol/l) reflected that of quintiles 1 and 2 in men (5-35 µmol/l and 36-46 µmol/l). Subsequently, quintiles 2 and 3 in women corresponded with plasma levels of quintiles 3 and 4 in men, respectively. In both men and women, plasma vitamin C levels were not significantly associated with heel bone density before and after adjustment for important confounding factors.

5.4.3.2 Secondary analysis

The impact of energy intake

Associations between vitamin C intake and bone density were also performed using vitamin C residuals in order to account for the total caloric consumption of participants. The results are presented in **Table 5.3**.

In men, the non-significant findings for crude dietary and total vitamin C intakes in association with BUA remained unchanged, although results for VOS differed slightly. Using residuals, the differences between the two upper quintiles of dietary vitamin C intake referent to quintile 1 were non-significant, although the trend for linearity was unchanged (β 2.13 m/s per quintile, *P*-trend=0.019). Associations between total intake and VOS were not affected by the use of residuals. In women, residuals of dietary vitamin C intake strengthened the associations with BUA, with both the trend for linearity and the differences between quintile 4 referent to quintile 1 being more significant. Results for total vitamin C intake and BUA were similar when using residuals and findings for dietary and total vitamin C in association with VOS remained non-significant.

Sensitivity analysis

Results from the primary analysis indicated that a large number of participants had very high plasma vitamin C concentrations. It was hypothesised that this may have resulted from the use of vitamin C supplements. Further analyses showed that the frequency of vitamin C supplement use increased significantly across quintiles of plasma vitamin C in both sexes ($P<0.001$), ranging from 12% to 55% in quintiles 1 to 5 in men respectively, and from 15% to 61% in women. Hence, a sensitivity analysis was performed in order to evaluate potential associations between vitamin C from the diet independent of supplement use.

Following the exclusion of current vitamin C supplement users **(Table 5.3)**, VOS in men and BUA in women remained significantly higher in those with higher dietary vitamin C intakes ($P \leq 0.008$). Interestingly, a significant 0.5% difference in VOS between extreme quintiles of dietary vitamin C intake referent to quintile 1 also became apparent in women (β 8.04 m/s, $P=0.024$). Plasma vitamin C concentrations were still not significantly associated with heel ultrasound in either sex.

The effects of body weight

Accounting for body weight did not change the associations between dietary vitamin C and bone density in men **(Table 5.3)**. However, the trend for linearity between VOS and total intake of vitamin C became significant when vitamin C intake was considered per kilogram bodyweight (β 2.38 m/s per quintile, *P*-trend=0.014). In contrast to men, body weight had a greater influence on associations in women. All significant associations between BUA and both dietary and total vitamin C intake lost significance when analysed per kilogram body weight. The non-significant relations between plasma vitamin C and heel bone density remained unchanged in both sexes.

Table 5.3: Associations between vitamin C and BUA and VOS in comparison to i) using residuals of vitamin C intake, ii) following the exclusion of vitamin C supplement users or iii) accounting for body weight.

Bone density stratified by vitamin C	**Men**				**Women**			
	n	**Q4 *vs.* Q1**	**Q5 *vs.* Q1**	**Linear trend**	***n***	**Q4 *vs.* Q1**	**Q5 *vs.* Q1**	**Linear trend**
Primary analysis								
BUA by dietary intake	968	β 1.85; *P*=0.31	β 0.39; *P*=0.83	β 0.35; *P*=0.39	1359	**β 2.59; *P*=0.038**	**β 4.06; *P*=0.001**	**β 0.82; *P*=0.004**
BUA by total intake	968	β 3.31; *P*=0.07	β 0.59; *P*=0.75	β 0.51; *P*=0.22	1359	**β 3.05; *P*=0.015**	**β 3.62; *P*=0.005**	**β 0.69; *P*=0.017**
BUA by plasma level	885	β 1.69; *P*=0.38	β -1.99; *P*=0.29	β -0.35; *P*=0.42	1195	β 0.33; *P*=0.80	β 0.83; *P*=0.53	β 0.12; *P*=0.69
VOS by dietary intake	968	**β 9.15; *P*=0.026**	**β 8.65; *P*=0.038**	**β 2.40; *P*=0.010**	1359	β -1.12; *P*=0.72	β 4.67; *P*=0.14	β 0.93; *P*=0.19
VOS by total intake	968	**β 12.95; *P*=0.002**	β 5.23; *P*=0.21	**β 2.01; *P*=0.034**	1359	β 2.10; *P*=0.51	β 5.43; *P*=0.09	β 0.78; *P*=0.28
VOS by plasma level	885	β 6.73; *P*=0.12	β -0.22; *P*=0.96	β 0.25; *P*=0.80	1195	β 2.86; *P*=0.37	β 0.01; *P*=1.00	β 0.28; *P*=0.71
Residuals of vitamin C								
BUA by dietary intake	967	β -0.08; *P*=0.97	β 0.05; *P*=0.98	β 0.32; *P*=0.42	1356	**β 2.98; *P*=0.015**	**β 3.67; *P*=0.003**	**β 0.86; *P*=0.002**
BUA by total intake	967	β 2.00; *P*=0.27	β 0.41; *P*=0.82	β 0.55; *P*=0.18	1356	β 2.31; *P*=0.07	**β 3.25; *P*=0.012**	**β 0.76; *P*=0.009**
VOS by dietary intake	967	β 2.83; *P*=0.49	β 5.29; *P*=0.19	**β 2.13; *P*=0.019**	1356	β -0.48; *P*=0.88	β 4.76; *P*=0.13	β 0.91; *P*=0.19
VOS by total intake	967	**β 8.19; *P*=0.044**	β 3.73; *P*=0.37	**β 2.02; *P*=0.028**	1356	β 3.03; *P*=0.33	β 4.60; *P*=0.15	β 1.22; *P*=0.09
Minus supplement users								
BUA by dietary intake	870	β 2.54; *P*=0.19	β 1.88; *P*=0.34	β 0.69; *P*=0.12	1113	**β 4.07; *P*=0.004**	**β 4.97; *P*<0.001**	**β 0.95; *P*=0.003**
BUA by plasma level	795	β 1.42; *P*=0.48	β -1.21; *P*=0.55	β -0.17; *P*=0.71	975	β 0.46; *P*=0.75	β 1.21; *P*=0.42	β 0.15; *P*=0.65
VOS by dietary intake	870	**β 13.26; *P*=0.002**	**β 11.60; *P*=0.008**	**β 3.08; *P*=0.002**	1113	β 0.82; *P*=0.82	**β 8.04; *P*=0.024**	β 1.37; *P*=0.09
VOS by plasma level	795	β 3.60; *P*=0.42	β 2.50; *P*=0.58	β 0.67; *P*=0.50	975	β 3.57; *P*=0.34	β 4.10; *P*=0.27	β 0.96; *P*=0.25
Vitamin C / body weight								
BUA by dietary intake	967	β 1.75; *P*=0.35	β -0.91; *P*=0.63	β 0.09; *P*=0.83	1356	β 1.55; *P*=0.22	β 2.49; *P*=0.06	β 0.53; *P*=0.07
BUA by total intake	967	β 2.50; *P*=0.18	β -0.01; *P*=1.00	β 0.34; *P*=0.43	1356	β 2.18; *P*=0.09	β 1.90; *P*=0.15	β 0.42; *P*=0.16
BUA by plasma level	884	β -1.08; *P*=0.58	β -2.39; *P*=0.48	β -0.43; *P*=0.35	1193	β 0.46; *P*=0.74	β -1.08; *P*=0.47	β -0.13; *P*=0.70
VOS by dietary intake	967	**β 10.08; *P*=0.017**	**β 8.48; *P*=0.045**	**β 2.46; *P*=0.010**	1356	β 0.48; *P*=0.88	**β 5.77; *P*=0.08**	β 1.11; *P*=0.13
VOS by total intake	967	**β 11.78; *P*=0.005**	β 6.55; *P*=0.12	**β 2.38; *P*=0.014**	1356	β 2.71; *P*=0.40	β 3.49; *P*=0.29	β 0.87; *P*=0.24
VOS by plasma level	884	β 4.12; *P*=0.34	β 5.91; *P*=0.20	β 1.41; *P*=0.18	1193	β 5.21; *P*=0.14	β 3.40; *P*=0.36	β 1.12; *P*=0.18

Total vitamin C intake is the sum of vitamin C intake from the diet and from supplements (mg/d). BUA in dB/MHz and VOS in m/s. All analyses were based on a multivariate-adjusted linear regression analysis.

5.4.4 Associations between vitamin C and fracture risk

5.4.4.1 Primary analysis

In the case-cohort sub-sample of EPIC-Norfolk participants, there were 112 hip fractures, 78 spine fractures and 70 wrist fractures in men, and 339 hip fractures, 124 spine fractures and 218 wrist fractures in women. In the case-cohort, which contained participants with a fracture at any of these three fracture sites, there were 248 and 616 total fractures in men and women, respectively. The results of the calculation of hazard ratios of fracture risk according to vitamin C intake or plasma concentrations are discussed in detail below. Briefly, higher plasma vitamin C concentrations were significantly inversely associated with the risk of hip, spine and total fractures in men only, and similar associations were also found between dietary intakes of vitamin C and total fracture risk in men only.

Vitamin C characteristics of participants with or without a fracture

Differences in dietary and total intake and plasma concentrations of vitamin C between those who did and did not fracture over the median 12.6-year follow-up are summarised in **Table 5.4**. Mean dietary or total vitamin C intake did not differ significantly between participants who had experienced a fracture at the hip, spine or wrist combined and those who stayed free from fractures. Similarly, mean plasma vitamin C levels did not differ significantly in women. However, mean plasma vitamin C concentrations were significantly lower in men with fractures compared to those who remained free from fractures (42.5±18.5 *vs.* 46.9±18.1 µmol/l, $P<0.001$).

Table 5.4: Vitamin C intake and plasma status in subjects with and without a total fracture.

	Subjects without a fracture				Subjects with a fracture				
	n	**Mean**	**(SD)**	**[Range]**	***n***	**Mean**	**(SD)**	**[Range]**	***P***
Men									
Dietary vitamin C intake (mg/d)	1709	85.8	(51.0)	[0; 471]	248	84.0	(57.5)	[0.9; 471]	0.60
Total vitamin C intake (mg/d)	1709	103.3	(113.2)	[0; 1595]	248	97.8	(117.6)	[0.9; 1202]	0.47
Plasma vitamin C levels (µmol/l)	1532	46.9	(18.1)	[3; 132]	222	42.5	(18.5)	[6; 94]	0.0008
Women									
Dietary vitamin C intake (mg/d)	2138	89.5	(49.8)	[0.2; 405]	616	88.2	(51.0)	[0.1; 353]	0.56
Total vitamin C intake (mg/d)	2138	125.6	(231.2)	[0.2; 6142]	616	119.2	(155.1)	[0.1; 1918]	0.52
Plasma vitamin C levels (µmol/l)	1851	58.4	(20.0)	[4; 170]	525	58.1	(20.2)	[5; 138]	0.75

Total vitamin C intake is the sum of vitamin C intake from foods and supplements.

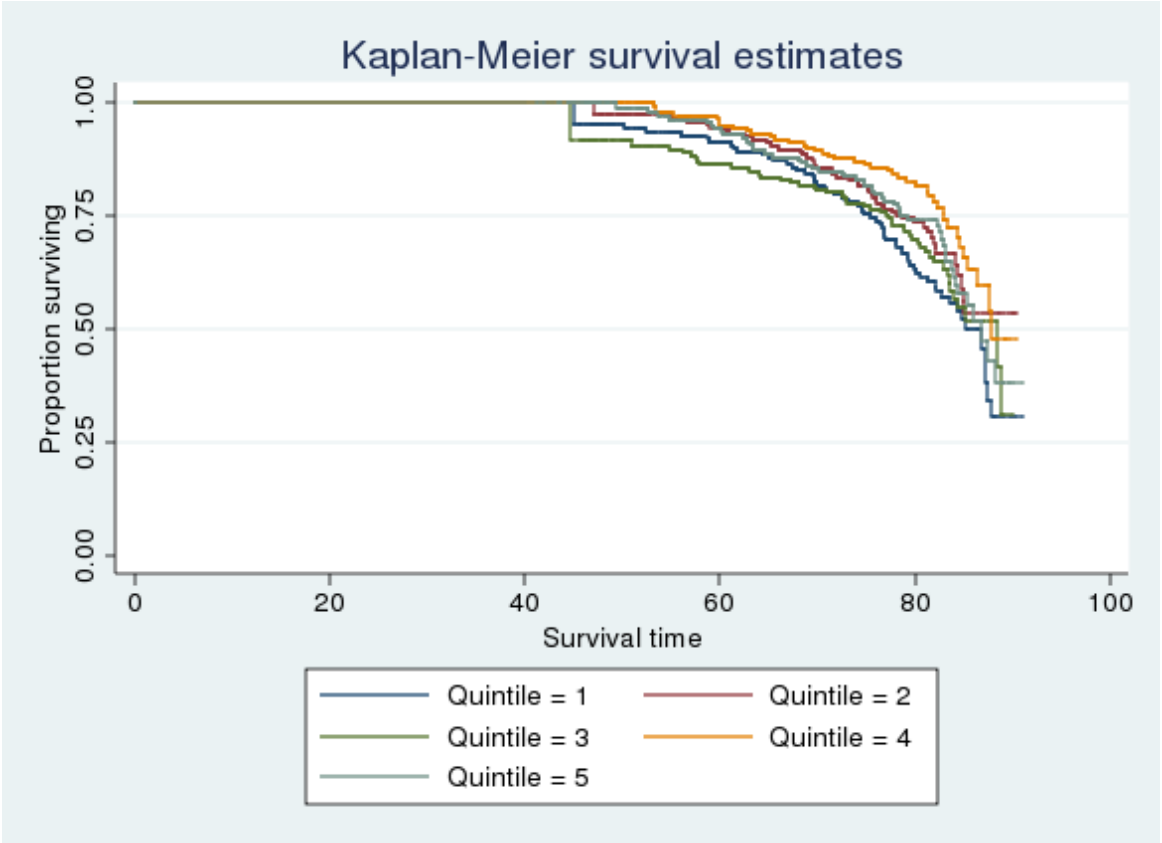
Dietary and total vitamin C intake and fracture risk

Pearson correlation coefficients for vitamin C intake from the diet and from the sum of dietary and supplemental (total) intake were 0.54 in men but only 0.31 in women ($P<0.05$).

In men, the Kaplan-Meier plot demonstrated some overlap and cross-over between quintiles of dietary vitamin C intake, although those in quintile 4 appeared to diverge markedly from those in the remaining quintiles for periods of time **(Figure 5.5)**. However, the log-rank test for equality showed that total osteoporotic fracture incidence did not differ significantly between quintiles of dietary vitamin C intake. Results from the Cox proportional hazard ratio analyses showed that the relationships between dietary vitamin C intake and fractures at the hip, spine and wrist were all non-linear **(Table 5.5)**. Although total osteoporotic fractures, describing the occurrence of fractures at the three different sites combined, showed a significant risk reduction for men in quintile 4 of dietary vitamin C intake compared to those in quintile 1, even after multivariate adjustment (HR 0.58, 95%CI 0.36-0.92; $P=0.020$). Results for total vitamin C intake were very similar **(Appendix 3, Table A3.1)**. Those men in quintile 4 of total vitamin C intake compared to those in quintile 1 had a significant reduction in total fracture risk (HR 0.52, 95%CI 0.33-0.83; $P=0.007$) and a marginally significant fracture risk reduction at the wrist (HR 0.41, 95%CI 0.17-0.99; $P=0.048$).

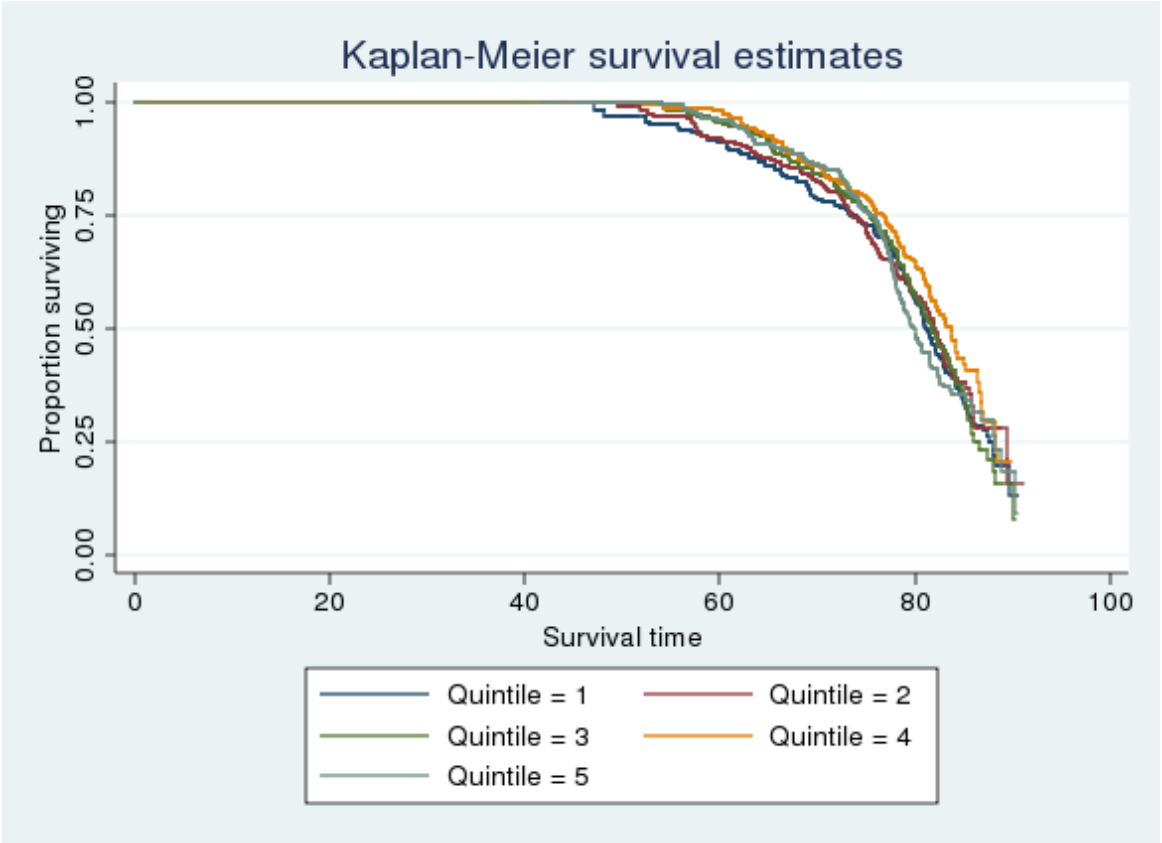
In women, the Kaplan-Meier plot predominantly showed both overlap and cross-over between quintiles of dietary vitamin C intake **(Figure 5.6)**, and the log rank test showed that there were no significant differences in total osteoporotic fracture incidence over the median 12.6-year follow-up between the different quintiles. Furthermore, Cox proportional hazard ratios revealed that dietary vitamin C intake **(Table 5.6)** as well as total vitamin C intake **(Appendix 3, Table A3.2)** were not associated with the risk of fracture at any site in EPIC-Norfolk women.

Figure 5.5: Kaplan-Meier plot of total fractures by quintiles of vitamin C intake in men.



The quintile groups differed significantly according to the log-rank test for equality (P=0.12). n=1957.

Figure 5.6: Kaplan-Meier plot of total fractures by quintiles of vitamin C intake in women.



There were no significant differences between the quintile groups according to the log-rank test for equality (P=0.32). n=2754.

Table 5.5: Associations between dietary vitamin C intake and fracture risk in men of the EPIC-Norfolk case-cohort.

		Dietary vitamin C intake (mg/d)								
		Quintile 1		**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		0 – 45.2		**45.3 – 61.9**		**62.0 – 85.6**		**85.7 – 119.6**		**119.7 - 471.4**	
		n = 392		***n = 391***		***n = 392***		***n = 391***		***n = 391***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[61]		[48]		[48]		[40]		[51]	
	Unadjusted	1.00	0.74	(0.48-1.13)	0.76	(0.50-1.15)	**0.59**	**(0.38-0.91)***	0.81	(0.53-1.22)	*P*=0.18
	Model 1	1.00	0.74	(0.48-1.13)	0.76	(0.50-1.15)	**0.60**	**(0.39-0.94)***	0.83	(0.55-1.26)	*P*=0.25
	Model 2	1.00	0.72	(0.47-1.11)	0.74	(0.48-1.13)	**0.58**	**(0.36-0.92)***	0.78	(0.51-1.21)	*P*=0.19
Hip fracture	[Events]	[30]		[21]		[24]		[18]		[19]	
	Unadjusted	1.00	0.67	(0.36-1.23)	0.77	(0.43-1.38)	0.54	(0.29-1.01)	0.61	(0.33-1.14)	*P*=0.09
	Model 1	1.00	0.68	(0.37-1.26)	0.77	(0.43-1.39)	0.55	(0.29-1.05)	0.64	(0.34-1.19)	*P*=0.12
	Model 2	1.00	0.68	(0.36-1.29)	0.77	(0.41-1.46)	0.55	(0.28-1.09)	0.64	(0.34-1.23)	*P*=0.15
Spinal fracture	[Events]	[19]		[12]		[15]		[11]		[21]	
	Unadjusted	1.00	0.59	(0.28-1.24)	0.75	(0.37-1.51)	0.52	(0.25-1.12)	1.10	(0.58-2.08)	*P*=0.86
	Model 1	1.00	0.58	(0.27-1.25)	0.75	(0.38-1.50)	0.52	(0.24-1.12)	1.12	(0.59-2.15)	*P*=0.80
	Model 2	1.00	0.57	(0.26-1.22)	0.72	(0.36-1.47)	0.50	(0.23-1.10)	1.05	(0.52-2.13)	*P*=0.91
Wrist fracture	[Events]	[16]		[17]		[12]		[10]		[15]	
	Unadjusted	1.00	1.06	(0.53-2.14)	0.75	(0.35-1.61)	0.62	(0.28-1.39)	0.94	(0.46-1.92)	*P*=0.47
	Model 1	1.00	1.06	(0.52-2.18)	0.75	(0.35-1.59)	0.64	(0.29-1.43)	0.95	(0.45-1.97)	*P*=0.52
	Model 2	1.00	0.96	(0.46-1.98)	0.63	(0.29-1.39)	0.56	(0.24-1.27)	0.79	(0.38-1.63)	*P*=0.28

*Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. Significant differences between the two upper quintiles referent to the lowest quintile: * (P<0.05), ** (P<0.01). Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity and use of steroids. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 1957 for total fracture, n 1842 for hip fracture, n 1808 for spine fracture, n 1806 for wrist fracture.*

Table 5.6: Associations between dietary vitamin C intake and fracture risk in women of the EPIC-Norfolk case-cohort.

		Dietary vitamin C intake (mg/d)								
		Quintile 1	**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		0.1 – 48.4	**48.5 – 69.9**		**67.0 – 90.5**		**90.6 – 124.2**		**124.3 – 405.3**	
		n = 551	***n = 551***		***n = 551***		***n = 551***		***n = 550***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[135]		[121]		[123]		[111]		[126]	
	Unadjusted	1.00	0.97	(0.72-1.32)	1.02	(0.75-1.37)	0.82	(0.61-1.12)	1.07	(0.79-1.45)	*P*=0.92
	Model 1	1.00	0.98	(0.72-1.34)	1.05	(0.77-1.43)	0.85	(0.62-1.15)	1.10	(0.81-1.50)	*P*=0.91
	Model 2	1.00	1.00	(0.74-1.37)	1.08	(0.79-1.47)	0.87	(0.64-1.20)	1.14	(0.83-1.58)	*P*=0.74
Hip fracture	[Events]	[71]		[67]		[68]		[60]		[73]	
	Unadjusted	1.00	1.09	(0.74-1.62)	1.08	(0.73-1.60)	0.93	(0.63-1.39)	1.26	(0.85-1.85)	*P*=0.49
	Model 1	1.00	1.11	(0.75-1.66)	1.16	(0.77-1.73)	0.98	(0.65-1.47)	1.26	(0.85-1.89)	*P*=0.45
	Model 2	1.00	1.15	(0.77-1.71)	1.20	(0.80-1.79)	1.02	(0.67-1.53)	1.33	(0.88-2.00)	*P*=0.34
Spinal fracture	[Events]	[36]		[20]		[21]		[21]		[26]	
	Unadjusted	1.00	0.60	(0.34-1.06)	0.62	(0.36-1.09)	0.61	(0.35-1.07)	0.81	(0.48-1.38)	*P*=0.44
	Model 1	1.00	0.62	(0.35-1.11)	0.67	(0.38-1.19)	0.64	(0.36-1.14)	0.88	(0.50-1.54)	*P*=0.62
	Model 2	1.00	0.63	(0.35-1.12)	0.68	(0.38-1.22)	0.65	(0.36-1.16)	0.90	(0.50-1.61)	*P*=0.68
Wrist fracture	[Events]	[51]		[42]		[46]		[37]		[42]	
	Unadjusted	1.00	0.83	(0.54-1.28)	0.94	(0.61-1.43)	0.70	(0.45-1.10)	0.85	(0.55-1.31)	*P*=0.33
	Model 1	1.00	0.82	(0.53-1.28)	0.94	(0.61-1.44)	0.69	(0.44-1.08)	0.84	(0.54-1.31)	*P*=0.30
	Model 2	1.00	0.84	(0.54-1.30)	0.95	(0.62-1.45)	0.69	(0.44-1.10)	0.84	(0.53-1.32)	*P*=0.30

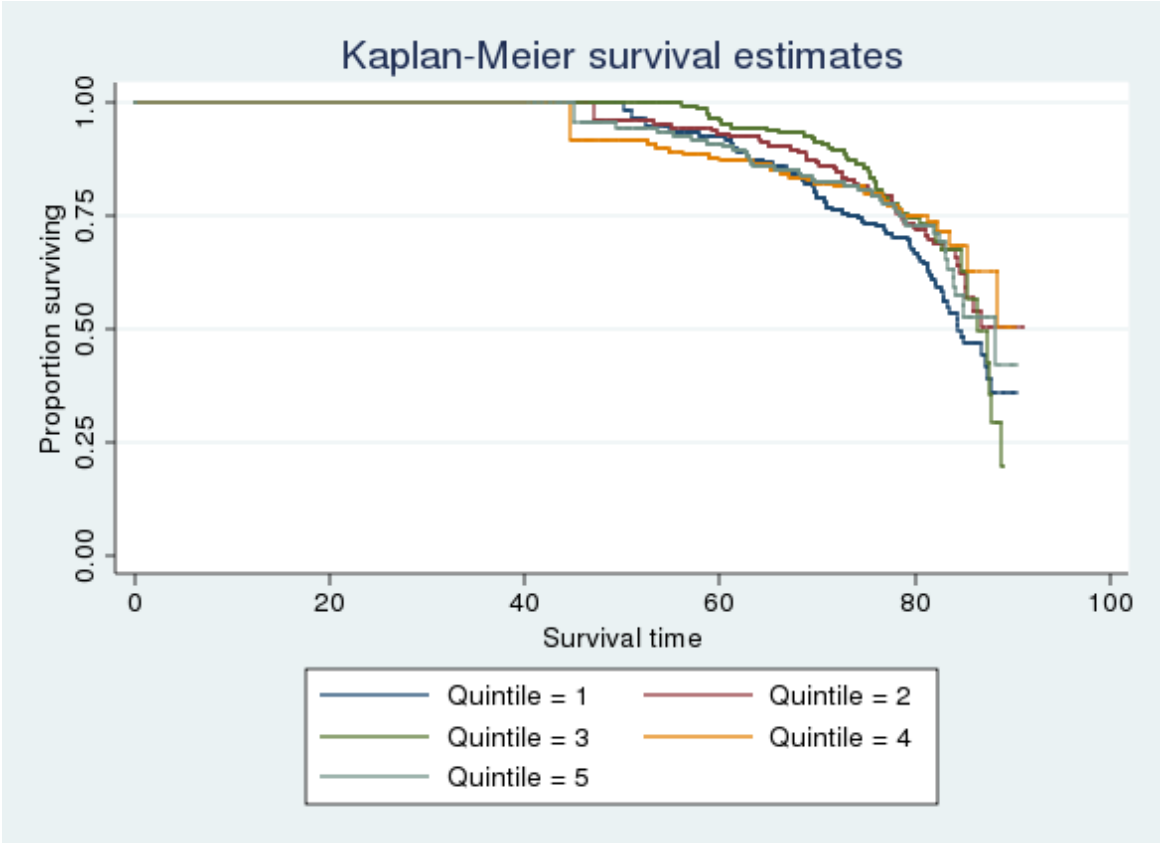
Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. No significant differences between the two upper quintiles referent to the lowest quintile. Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids, menopausal status and HRT. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 2754 for total fracture, n 2525 for hip fracture, n 2334 for spine fracture, n 2409 for wrist fracture.

Plasma vitamin C concentrations and fracture risk

In the 1754 men, there was no clear divergence between quintiles of plasma vitamin C in relation to total osteoporotic fracture incidence as shown by the Kaplan-Meier plot **(Figure 5.7)**, although results from the log-rank test for equality indicated that the quintiles differed marginally significantly (*P*=0.047). In concordance with these findings, results from the Cox proportional hazard ratio analyses showed that there was a linear inverse relationship between plasma vitamin C levels and hip fractures (HR 0.82, *P*-trend=0.016), and a marginally significant relationship with total osteoporotic fractures (HR 0.89, *P*-trend=0.046) after adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroid medication, energy intake, dietary calcium intake and the use of calcium and vitamin D supplements **(Table 5.7)**. Moreover, men in quintile 4 of plasma vitamin C had a significantly lower risk of hip fracture (HR 0.35, 95%CI 0.16-0.80; *P*=0.012), spine fracture (HR 0.26, 95%CI 0.10-0.69; *P*=0.007) and total osteoporotic fracture (HR 0.50, 95%CI 0.31-0.82; *P*=0.006) compared to those in the lowest quintile, even after adjustment for important confounding factors. Wrist fractures were not significantly associated with plasma vitamin C concentrations in EPIC-Norfolk men.

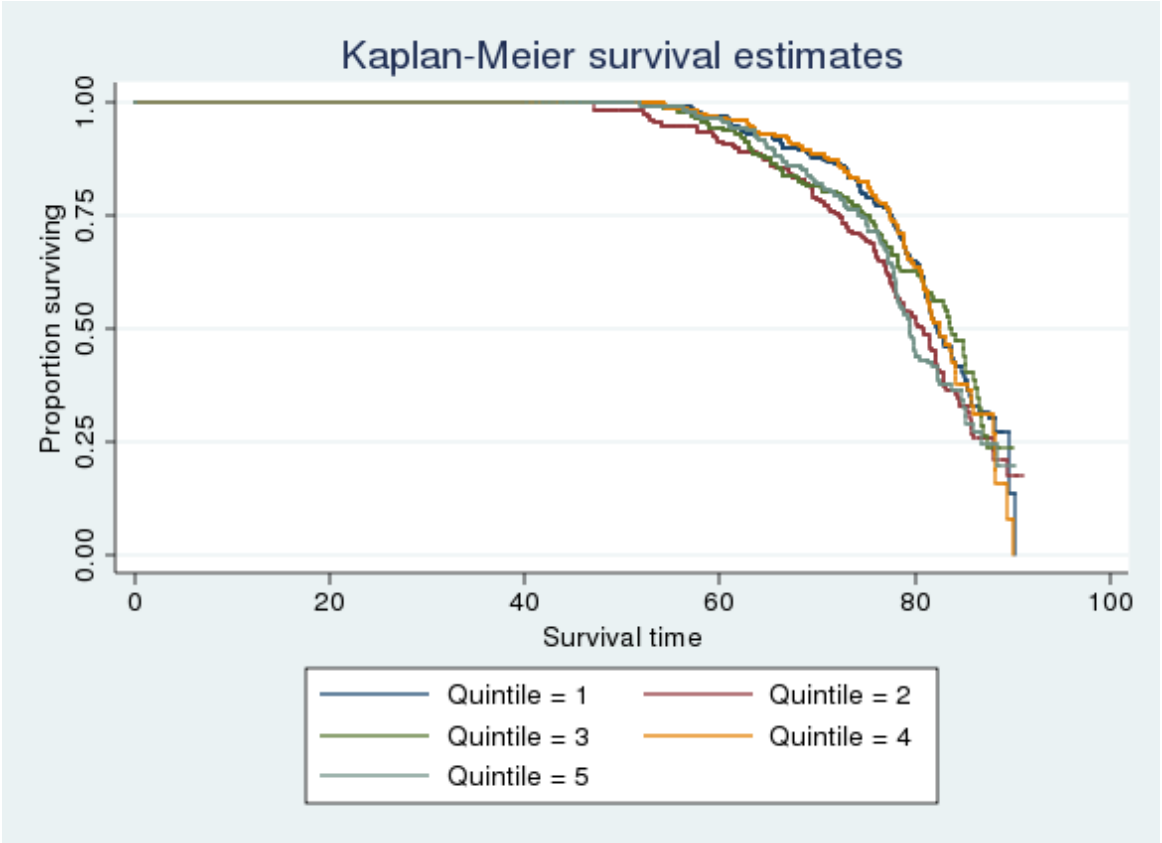
In the 2376 women, the log-rank test for equality showed no significant differences in total osteoporotic fracture incidence over the 11-year follow-up between the different quintiles of plasma vitamin C levels, and this was confirmed by quintile overlap and cross-over in the Kaplan-Meier plot **(Figure 5.8)**. Furthermore, results from the multiple Cox regression analyses showed that there were no linear relationships between plasma vitamin C concentrations and risk of fracture at all measured sites before and after adjustment for important confounding factors **(Table 5.8)**. There were also no associations between the top two quintiles referent to the lowest quintile of plasma vitamin C in these women.

Figure 5.7: Kaplan-Meier plot of total fractures by quintiles of plasma vitamin C in men.



* *The quintile groups differed significantly according to the log-rank test for equality (P=0.047). n=1754.*

Figure 5.8: Kaplan-Meier plot of total fractures by quintiles of plasma vitamin C in women.



* *There were no significant differences between the quintile groups according to the log-rank test for equality (P=0.09). n=2376.*

Table 5.7: Associations between plasma vitamin C and fracture risk in men of the EPIC-Norfolk case-cohort.

		Plasma vitamin C (µmol/l)									
		Quintile 1		**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		3 - 30		**31 - 43**		**44 - 52**		**53 - 61**		**62 - 132**	
		n = 372		***n = 388***		***n = 383***		***n = 336***		***n = 363***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Event]	[65]		[46]		[44]		[27]		[40]	
	Unadjusted	1.00	0.70	(0.46-1.06)	0.80	(0.52-1.24)	**0.52**	**(0.32-0.84)****	0.71	(0.45-1.10)	*P*=0.06
	Model 1	1.00	0.70	(0.45-1.08)	0.76	(0.49-1.19)	**0.50**	**(0.30-0.81)****	0.69	(0.43-1.09)	***P*=0.048**
	Model 2	1.00	0.71	(0.46-1.09)	0.76	(0.49-1.19)	**0.50**	**(0.31-0.82)****	0.68	(0.42-1.08)	***P*=0.046**
Hip fracture	[Event]	[33]		[23]		[20]		[8]		[14]	
	Unadjusted	1.00	0.77	(0.43-1.38)	0.88	(0.47-1.62)	**0.36**	**(0.16-0.81)***	0.56	(0.29-1.11)	***P*=0.025**
	Model 1	1.00	0.78	(0.42-1.43)	0.78	(0.42-1.48)	**0.34**	**(0.15-0.77)****	0.51	(0.25-1.04)	***P*=0.013**
	Model 2	1.00	0.77	(0.42-1.43)	0.76	(0.40-1.42)	**0.35**	**(0.16-0.80)***	0.52	(0.25-1.06)	***P*=0.016**
Spinal fracture	[Event]	[21]		[14]		[12]		**[5]**		[17]	
	Unadjusted	1.00	0.71	(0.35-1.41)	0.67	(0.32-1.41)	**0.30**	**(0.11-0.80)***	0.97	(0.50-1.90)	*P*=0.49
	Model 1	1.00	0.70	(0.34-1.42)	0.61	(0.28-1.33)	**0.28**	**(0.10-0.75)***	0.95	(0.46-1.98)	*P*=0.47
	Model 2	1.00	0.68	(0.33-1.40)	0.61	(0.28-1.32)	**0.26**	**(0.10-0.69)****	0.90	(0.42-1.90)	*P*=0.38
Wrist fracture	[Event]	[16]		[10]		[14]		[15]		[11]	
	Unadjusted	1.00	0.62	(0.28-1.39)	0.89	(0.43-1.88)	1.01	(0.49-2.10)	0.70	(0.32-1.53)	*P*=0.77
	Model 1	1.00	0.62	(0.27-1.41)	0.89	(0.41-1.92)	1.02	(0.47-2.18)	0.72	(0.32-1.60)	*P*=0.83
	Model 2	1.00	0.65	(0.28-1.49)	0.95	(0.43-2.07)	1.12	(0.52-2.42)	0.77	(0.34-1.72)	*P*=1.00

*Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. Significant differences between the two upper quintiles referent to the lowest quintile: * (P<0.05), ** (P<0.01). Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity and use of steroids. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 1754 for total fracture, n 1650 for hip fracture, n 1619 for spine fracture, n 1621 for wrist fracture.*

Table 5.8: Associations between plasma vitamin C and fracture risk in women of the EPIC-Norfolk case-cohort.

		Plasma vitamin C (µmol/l)									
		Quintile 1	**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**		
		4 - 43	**44 - 54**		**55 - 63**		**64 - 73**		**74 - 170**		
		n = 533	***n = 497***		***n = 518***		***n = 527***		***n = 479***		
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Event]	[109]		[122]		[88]		[97]		[109]	
	Unadjusted	1.00	1.26	(0.92-1.73)	1.00	(0.71-1.40)	1.03	(0.74-1.43)	1.33	(0.96-1.84)	*P*=0.34
	Model 1	1.00	1.29	(0.94-1.78)	0.98	(0.69-1.39)	1.02	(0.73-1.42)	1.33	(0.95-1.85)	*P*=0.41
	Model 2	1.00	1.30	(0.94-1.80)	1.00	(0.70-1.42)	1.04	(0.74-1.46)	1.35	(0.96-1.90)	*P*=0.33
Hip fracture	[Event]	[59]		[61]		[52]		[57]		[62]	
	Unadjusted	1.00	1.18	(0.78-1.80)	1.14	(0.74-1.76)	1.18	(0.78-1.80)	1.48	(0.97-2.25)	*P*=0.11
	Model 1	1.00	1.19	(0.78-1.82)	1.08	(0.69-1.68)	1.11	(0.71-1.73)	1.43	(0.93-2.21)	*P*=0.20
	Model 2	1.00	1.20	(0.79-1.84)	1.09	(0.69-1.72)	1.13	(0.73-1.77)	1.46	(0.94-2.27)	*P*=0.18
Spinal fracture	[Event]	[22]		[32]		[19]		[22]		[15]	
	Unadjusted	1.00	1.71	(0.96-3.04)	0.98	(0.52-1.85)	1.15	(0.62-2.13)	0.89	(0.45-1.77)	*P*=0.41
	Model 1	1.00	1.80	(1.01-3.23)	1.01	(0.52-1.97)	1.20	(0.63-2.32)	0.97	(0.47-1.99)	*P*=0.59
	Model 2	1.00	1.83	(1.02-3.29)	1.03	(0.52-2.03)	1.24	(0.63-2.42)	1.01	(0.48-2.11)	*P*=0.71
Wrist fracture	[Event]	[39]		[38]		[32]		[33]		[40]	
	Unadjusted	1.00	1.09	(0.68-1.74)	1.00	(0.61-1.64)	0.94	(0.58-1.53)	1.26	(0.79-2.00)	*P*=0.56
	Model 1	1.00	1.10	(0.69-1.77)	0.96	(0.58-1.59)	0.89	(0.54-1.47)	1.19	(0.74-1.91)	*P*=0.80
	Model 2	1.00	1.13	(0.70-1.82)	0.98	(0.59-1.63)	0.91	(0.55-1.51)	1.19	(0.73-1.93)	*P*=0.80

Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. No significant differences between the two upper quintiles referent to the lowest quintile. Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids, menopausal status and HRT. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 2376 for total fracture, n 2183 for hip fracture, n 2024 for spine fracture, n 2080 for wrist fracture.

5.4.4.2 Secondary analysis

The impact of energy intake

As shown in **Table 5.9**, results for Cox proportional hazards of total fracture risk using residuals of both dietary and total vitamin C intake did not differ from findings of crude vitamin C intakes in women. However, the reduction in total fracture risk for men in quintile 4 of dietary as well as total intake compared to quintile 1 became non-significant.

Sensitivity analysis

The exclusion of participants reporting the current use of vitamin C supplements did not affect the present findings. As shown in **Table 5.9**, there were still no associations between vitamin C and total fracture risk in women, and all significant risk reductions in men with both dietary intake and plasma levels of vitamin C remained significant. However, interestingly, the significant total fracture risk reduction for plasma vitamin C quintile 4 referent to quintile 1 of the crude analysis (HR 0.50, 95%CI 0.31-0.82; *P*=0.006) became even more significant (HR 0.38, 95%CI 0.22-0.65; *P*<0.001).

The effects of body weight

As shown in **Table 5.9**, when dietary vitamin C intake was considered per kilogram bodyweight, the significant reduction in total fracture risk for men in quintile 4 referent to quintile 1 became non-significant. Results for total intake per kilogram bodyweight remained unchanged in comparison to the crude analyses. However, there was a marginally significant total fracture risk reduction between extreme quintiles of plasma vitamin C (HR 0.60, 95%CI 0.36-0.99; *P*=0.046) which was not significant in the crude analyses. In women, the non-significant findings between intake and plasma levels of vitamin C and total fracture risk remained unchanged when body weight was accounted for.

Associations in older people

Table 5.9 also shows the relationship between vitamin C and total fracture risk in older men and women aged 65 years and over at baseline. Findings for dietary intakes of vitamin C were similar to those of the full case-cohort sample in both sexes, although there were no significant associations between total intake and total fracture risk in the older men. Furthermore, the significant linear inverse relationship between plasma vitamin C and total fracture risk in men was no longer significant when younger participants were excluded from the analyses.

Table 5.9: Associations between vitamin C and total fracture risk in comparison to i) using residuals of vitamin C intake, ii) following the exclusion of vitamin C supplement users, iii) accounting for body weight or iv) investigations undertaken in the elderly population only.

Total fracture risk stratified by vitamin C	**Men**				**Women**			
	***n* cases/ non-cases**	**Q4 *vs.* Q1**	**Q5 *vs.* Q1**	**Linear trend**	***n* cases/ non-cases**	**Q4 *vs.* Q1**	**Q5 *vs.* Q1**	**Linear trend**
Primary analysis								
Dietary intake	248/1709	**HR 0.58; *P*=0.020 (95%CI 0.36-0.92)**	HR 0.78; *P*=0.27 (95%CI 0.51-1.21)	HR 0.93; *P*=0.19 (95%CI 0.84-1.04)	616/2138	HR 0.87; *P*=0.40 (95%CI 0.64-1.20)	HR 1.14; *P*=0.41 (95%CI 0.83-1.58)	HR 1.01; *P*=0.74 (95%CI 0.94-1.09)
Total intake	248/1709	**HR 0.52; *P*=0.007 (95%CI 0.33-0.83)**	HR 0.76; *P*=0.23 (95%CI 0.49-1.18)	HR 0.92; *P*=0.15 (95%CI 0.83-1.03)	616/2138	HR 0.99; *P*=0.97 (95%CI 0.72-1.37)	HR 1.16; *P*=0.40 (95%CI 0.83-1.61)	HR 1.02; *P*=0.66 (95%CI 0.94-1.10)
Plasma concentrations	222/1532	**HR 0.50; *P*=0.006 (95%CI 0.31-0.82)**	HR 0.68; *P*=0.10 (95%CI 0.42-1.08)	**HR 0.89; *P*=0.046 (95%CI 0.80-1.00)**	525/1851	HR 1.04; *P*=0.81 (95%CI 0.74-1.46)	HR 1.35; *P*=0.08 (95%CI 0.96-1.90)	HR 1.04; *P*=0.33 (95%CI 0.96-1.13)
Residuals of vitamin C								
Dietary intake	248/1709	HR 0.66; *P*=0.06 (95%CI 0.42-1.02)	HR 0.78; *P*=0.26 (95%CI 0.51-1.20)	HR 0.94; *P*=0.26 (95%CI 0.85-1.04)	616/2138	HR 0.86; *P*=0.36 (95%CI 0.63-1.18)	HR 1.17; *P*=0.33 (95%CI 0.85-1.60)	HR 1.01; *P*=0.83 (95%CI 0.94-1.08)
Total intake	248/1709	HR 0.66; *P*=0.07 (95%CI 0.43-1.03)	HR 0.71; *P*=0.13 (95%CI 0.46-1.10)	HR 0.93; *P*=0.18 (95%CI 0.84-1.03)	616/2138	HR 1.05; *P*=0.77 (95%CI 0.76-1.45)	HR 1.09; *P*=0.61 (95%CI 0.78-1.52)	HR 1.02; *P*=0.56 (95%CI 0.95-1.10)
Minus supplement users								
Dietary intake	228/1780	**HR 0.54; *P*=0.015 (95%CI 0.33-0.89)**	HR 0.76; *P*=0.23 (95%CI 0.48-1.20)	HR 0.91; *P*=0.11 (95%CI 0.81-1.02)	525/2309	HR 0.95; *P*=0.76 (95%CI 0.67-1.34)	HR 1.16; *P*=0.40 (95%CI 0.82-1.63)	HR 1.03; *P*=0.50 (95%CI 0.95-1.11)
Plasma concentrations	203/1596	**HR 0.38; *P*<0.001 (95%CI 0.22-0.65)**	HR 0.67; *P*=0.10 (95%CI 0.41-1.08)	**HR 0.87; *P*=0.018 (95%CI 0.77-0.98)**	445/1983	HR 1.31; *P*=0.14 (95%CI 0.91-1.89)	HR 1.17; *P*=0.41 (95%CI 0.80-1.72)	HR 1.04; *P*=0.33 (95%CI 0.96-1.13)
Vitamin C / body weight								
Dietary intake	248/1709	HR 0.70; *P*=0.13 (95%CI 0.44-1.11)	HR 0.78; *P*=0.29 (95%CI 0.50-1.23)	HR 0.94; *P*=0.22 (95%CI 0.84-1.04)	616/2138	HR 0.96; *P*=0.80 (95%CI 0.70-1.32)	HR 1.07; *P*=0.70 (95%CI 0.76-1.50)	HR 1.01; *P*=0.88 (95%CI 0.93-1.09)
Total intake	248/1709	**HR 0.59; *P*=0.032 (95%CI 0.36-0.96)**	HR 0.74; *P*=0.20 (95%CI 0.47-1.17)	HR 0.93; *P*=0.21 (95%CI 0.83-1.04)	616/2138	HR 1.03; *P*=0.85 (95%CI 0.75-1.43)	HR 1.19; *P*=0.33 (95%CI 0.84-1.68)	HR 1.03; *P*=0.41 (95%CI 0.95-1.12)
Plasma concentrations	222/1532	**HR 0.61; *P*=0.03 (95%CI 0.39-0.95)**	**HR 0.60; *P*=0.046 (95%CI 0.36-0.99)**	**HR 0.87; *P*=0.017 (95%CI 0.77-0.97)**	525/1851	HR 1.17; *P*=0.40 (95%CI 0.81-1.67)	HR 1.23; *P*=0.29 (95%CI 0.84-1.79)	HR 1.04; *P*=0.37 (95%CI 0.95-1.14)
Subjects aged 65+ years								
Dietary intake	131/587	**HR 0.48; *P*=0.035 (95%CI 0.24-0.95)**	HR 0.64; *P*=0.15 (95%CI 0.34-1.18)	HR 0.89; *P*=0.15 (95%CI 0.76-1.04)	390/633	HR 0.89; *P*=0.60 (95%CI 0.58-1.38)	HR 1.20; *P*=0.41 (95%CI 0.77-1.86)	HR 1.04; *P*=0.44 (95%CI 0.94-1.15)
Total intake	131/587	HR 0.56; *P*=0.09 (95%CI 0.29-1.09)	HR 0.64; *P*=0.18 (95%CI 0.33-1.22)	HR 0.89; *P*=0.16 (95%CI 0.76-1.04)	390/633	HR 1.06; *P*=0.80 (95%CI 0.69-1.63)	HR 1.17; *P*=0.74 (95%CI 0.74-1.85)	HR 1.04; *P*=0.49 (95%CI 0.94-1.15)
Plasma concentrations	119/519	**HR 0.37; *P*=0.012 (95%CI 0.17-0.81)**	HR 0.76; *P*=0.41 (95%CI 0.39-1.46)	HR 0.88; *P*=0.12 (95%CI 0.75-1.03)	330/544	HR 1.28; *P*=0.30 (95%CI 0.80-2.04)	HR 1.12; *P*=0.65 (95%CI 0.69-1.81)	HR 1.05; *P*=0.43 (95%CI 0.94-1.17)

Total vitamin C intake is the sum of vitamin C intake from the diet and from supplements (mg/d). The analyses are adjusted Prentice-weighted Cox proportional hazard ratios of total fracture risk.

5.5 Discussion

To my knowledge, these data are the first to investigate potential cross-sectional and prospective associations between intakes and plasma levels of vitamin C and measures of bone density as well as fracture risk in the same population group in both men and women. Although prospective associations between vitamin C intake and status with bone density have previously been published on this cohort [166], the study by Kaptoge *et al.* focused on 2-5 year change in BMD measured by DXA in a small sub-cohort of 944 participants aged 67-79 years and they did not adjust for a number of important confounding factors including age, sex and smoking status. In contrast, the present study investigated both cross-sectional associations with heel ultrasound and prospective associations with fracture risk, adjusting for more confounding factors, and was undertaken in a much larger sub-cohort of up to 5319 participants with a much wider age range (39-79 years). Following multivariate adjustment, the results from the primary analysis showed that i) dietary and total intakes of vitamin C were significantly and positively associated with either BUA or VOS measures of heel ultrasound in men and women, and ii) higher intakes of vitamin C, but more so plasma concentrations, were significantly and inversely associated with the risk of fragility fractures in men only.

5.5.1 Heel ultrasound

In men, there was a linear relationship between vitamin C intakes and VOS, with dietary intakes of 92-127 mg/d and 127-471 mg/d being associated with 0.6% higher VOS compared to intakes of up to 48 mg/d. In women, dietary intakes of 96-129 mg/d and 129-353 mg/d were significantly associated with 3.2-4.2% higher BUA compared to intakes of up to 52 mg/d, and this relationship was also found to be linear. In both sexes, the differences in mean dietary vitamin C intake between the two upper quintile groups compared to the lowest quintile were approximately 70 mg/d for quintile 4 and 136 mg/d for quintile 5. These higher vitamin C intakes are easily achievable through the usual diet and may reflect the consumption of, for example, one orange per day to reach the levels of intake for quintile 4 and one orange as well as three average-sized broccoli spears per day for quintile 5 [145, 350]. The results for total vitamin C intake, derived from the sum of vitamin C intake from the diet and from supplements, were very similar to those for dietary intake in women and mostly comparable to those in men. The comparability might be explained by the high level of agreement in the classification of participants into sex-specific quintiles of dietary and total vitamin C intake. Residual adjustment of dietary and total intake had little influence on these findings. The cross-sectional findings of a positive association between vitamin C intake and measures of heel ultrasound potentially reflect the important role of vitamin C in bone health. It is well documented that vitamin C plays a crucial role as a cofactor in the hydroxylation reactions within collagen fibres [7-10], and this increases overall collagen

strength [136]. Moreover, recent cell and animal studies have reported that vitamin C may also mediate osteoclastogenesis and osteoblastogenesis [161-164], although the precise biological mechanisms have not been fully established yet. Furthermore, vitamin C may also be one explanatory factor for the positive associations found between fruit and vegetable intakes and bone health in this cohort. Vitamin C is exclusively found in fruits and vegetables [145, 225], and positive associations between their intakes and measures of bone health were reported in previous epidemiological studies [90, 131-134], although the underlying mechanisms are not fully established yet. Preliminary analyses in this cohort presented in Chapter 3 (pages 69-71) showed that higher *vs.* lower intakes of vegetables were significantly associated with 0.7% higher VOS in men (compared to 0.6% for vitamin C) and fruit and vegetable intakes with 5.1-7.3% higher BUA in women (compared to 3.2-4.4% for vitamin C).

In contrast to diet, vitamin C status was not found to be associated with heel bone density in men and women of the present study. Dietary intakes and plasma concentrations of vitamin C may not have shown comparable results because the ranking of subjects into sex-specific quintiles of plasma vitamin C showed some disagreement with both dietary and total intakes of vitamin C (this will be discussed in more detail in Chapter 8, page 212). To my knowledge, only one previous study from the US has investigated the cross-sectional relationship between vitamin C status and BMD in a large population of 13080 men and women [169]. The results were sex-specific and showed that men with serum vitamin C concentrations of 28.4-56.8 µmol/l had higher hip BMD compared to men with lower or higher blood levels, but no such observations were made in women. As vitamin C status measures can overcome issues associated with measuring intake including recall error and bioavailability [170] and current data on the relationship between blood vitamin C levels and bone density is scarce, more cross-sectional studies using vitamin C status are needed.

Percentage differences in VOS were much smaller than those of BUA in the present study, possibly due to the scale differences between these two bone parameters. However, one previous study has shown that their relative fracture risk implications are very similar [66]. Moreover, the magnitude of effect of BUA is in agreement with the literature, as effect sizes of 3-4.5% for hip and lumbar spine BMD have previously been found with higher intakes of vitamin C [133]. The percentage difference in BUA between low and high intakes of vitamin C as reported in the present study was also comparable to effect sizes previously reported for other bone-related dietary factors including intakes of potassium (3-4%) and magnesium (3%) [133]. To date, there is no cross-sectional data that used dietary vitamin C intakes and VOS measurements, and associations between dietary intakes of other nutrients and VOS have either not been significant [290] or were not reported in great enough detail [351]. Hence, the effect size of 0.6% for men with higher dietary vitamin C intakes as reported in the present study could not be compared to previous studies, suggesting that it is a novel finding.

It has previously been reported that supplement users compared to non-users tend to consume a more micronutrient-rich diet [262, 348, 349]. Thus, we also performed our analyses following the exclusion of participants with self-reported current use of vitamin C supplements, although this did not affect the present findings significantly.

Nonetheless, a 0.4% marginally significant and positive difference in VOS between extreme quintiles of dietary vitamin C intake was revealed in women, which was not found in the primary analysis. There is currently no data in the literature on the relationship between nutrients from the diet and VOS in women. However, in the present primary analyses, associations between vitamin C and heel ultrasound in women have been consistently found for BUA rather than VOS. It is thus reasonable to assume that this secondary finding, although beneficial, may have occurred by chance.

Associations between intake and status of vitamin C were mainly independent of body weight in men, although body weight had a greater influence in women. For example, all significant relationships between vitamin C intake and BUA in women lost significance; whereas total intake gained significance in relation to VOS. As previously discussed, the latter may have been a chance finding.

Interestingly, in the present study, vitamin C intakes were associated with VOS in men and BUA in women. Potential reasons for this sex difference are currently not known. However, there is evidence regarding the independent heritability of the two ultrasound parameters [77], and both measures have also been shown to be independently associated with osteoporotic fractures [21, 69, 70].

5.5.2 Fracture risk

To my knowledge, the potential prospective relationship between vitamin C and the risk of fractures has not previously been investigated in a British population. Using data from the EPIC-Norfolk cohort, the present novel findings showed that men with higher dietary vitamin C intakes (86-199 mg/d) had a 48% lower total fracture risk compared to those with the lowest intakes (0-45 mg/d) after a median of 12.6-years follow-up. In line with vitamin C being exclusively found in plant-based foods [145, 225], this effect size is smaller than those of our preliminary analyses presented in Chapter 3 (pages 69-71), which showed a 69% and 57% reduction in hip fracture risk in men with higher *vs.* lower intakes of fruit and of fruit and vegetables, respectively. Total vitamin C intakes showed further fracture risk reductions at the wrist for men with intakes of 92-130 mg/d compared to 0-46 mg/d. Vitamin C was not a significant predictor of fracture risk in women, despite the significant inverse associations between intakes of fruit and spine fracture risk in this cohort, as shown in Chapter 3. Residual adjustment of dietary and total vitamin C intake did not affect the significant study outcomes. In comparison to the present findings, only one US-based prospective cohort study investigated

fracture risk in relation to vitamin C intake and the authors reported bone-site specific and vitamin C intake-specific associations [167]. For example, no significant associations were found between dietary vitamin C intake and fracture risk at any site; whereas total vitamin C intake was associated with a 44% reduction in fracture risk at the hip. Despite the longer follow-up of 15-17 years, this study may have reported non-significant results for dietary vitamin C due to a small sample size of 918 participants and the analyses of a combined sample of men and women. Furthermore, the use of tertiles of vitamin C intake may not have given a fine enough discrimination of the data.

Potential prospective associations between vitamin C status and fracture risk have not previously been investigated, and the present study indicated low blood levels were a significant predictor of fracture risk in men. We found that the mean plasma vitamin C concentration of 42.5 µmol/l of men with a fracture was significantly lower than 46.9 µmol/l of those who remained free from fractures after the median 12.6-year follow-up. Moreover, men with plasma levels of 53-61 µmol/l had a significant fracture risk reduction of 65% at the hip, 74% at the spine and 52% for total osteoporotic fractures compared to men with blood levels of up to 30 µmol/l. Furthermore, hip and total osteoporotic fracture risk was inversely related to plasma vitamin C in a linear fashion. In women, there was no association between plasma vitamin C concentrations and fracture risk at any site. The reasons for this are unclear, the number of fractures did not differ across the quintile groups of plasma vitamin C in women. As the present study was the first of its kind to investigate potential prospective associations between vitamin C status and fracture risk, more prospective cohort studies are needed to confirm the present findings and to help understand whether the beneficial results observed in men are indeed sex-specific.

The present prospective findings were not affected by age or by the current use of vitamin C supplements but were significantly influenced by body weight. For example, the associations in men between dietary vitamin C intake and total osteoporotic fracture risk weakened, whereas that for both total intake and plasma levels of vitamin C lost significance when vitamin C intake and plasma status was assessed according to body weight. Reasons for this may include the influence of body weight on the distribution of vitamin C in the body.

5.5.3 Strengths and limitations

The present study offers a number of potential advances over previous observational studies. The inclusion of men and women in the study population provided more evidence that the potential beneficial effects of higher vitamin C intake for greater bone density and a reduction in fracture risk are relevant to both sexes. There have only been two cross-sectional studies and three prospective studies that examined associations in populations of both sexes, however positive results were often restricted to population subgroups. For example, associations

between dietary vitamin C intake and bone density have been reported for pre-menopausal women only [169] and another study found that total vitamin C intake was associated with bone density only among male never-smokers [230]. Furthermore, the only prospective study investigating associations between vitamin C and fracture risk used a combined sample of men and women rather than performing sex-specific analyses. Hence, the sole stratification by vitamin C quintile groups and by sex make the present study novel in providing evidence for a positive association between vitamin C intake and bone density and an inverse association between plasma vitamin C and fracture risk in a representative UK population. Moreover, the present cross-sectional and prospective studies, comprising a case-cohort sample of up to 5011 men and women of the EPIC-Norfolk cohort, addressed previous limitations regarding small sample sizes. To date, only two US-based cross-sectional studies encompassed cohorts of more than 11,000 [342] and 13,000 subjects [169]; whereas all other observational studies comprised cohorts of less than 1000 participants **(Appendix 2, Tables A2.2-A2.4)**. Investigations into the potential relationship between intakes and status levels of vitamin C and bone health have not previously been conducted in such a large cohort of older British men and women. Thus the present observational studies may provide novel data for the UK of a representative Caucasian population.

Although the present study had a robust study design, it also had a number of limitations. For example, the cross-sectional study design of the bone density analyses only examined relations between diet and bone density for a single point in time. The positive associations found in this study suggest that there was a relation between dietary and total vitamin C intakes and heel bone density in both men and women, but conclusions about the influence of vitamin C on bone health cannot be drawn. Similarly, the prospective study design of the fracture analyses was limited by the inability to identify possible secular changes in dietary vitamin C intakes and plasma vitamin C concentrations over the follow-up period and subsequent exposure misclassification, as data were only available from the 7-day food diaries and blood sample collected at baseline. Another limitation to the present study may have been the high mean vitamin C intake of the study population. Although dietary vitamin C intakes in this cohort (86 mg/d in men and 89 mg/d in women) were comparable to those of the general UK population aged 50-64 years around the time of data collection (94.5 mg/d) [292]; around 87% of the EPIC-Norfolk population had higher intakes of vitamin C than the UK RNI of 40 mg/d [225]. Hence, in the present study, associations between extreme quintiles of vitamin C and bone parameters may have been weakened by the population's overall high dietary intake of the nutrient. Although multivariate adjustment models were applied to the analyses, a number of other relevant confounders previously associated with bone health including sunlight exposure [295] were not measured as part of the EPIC-Norfolk study. Furthermore, residual confounding

may have occurred despite the multivariate modelling and may have resulted in bias in exposure effect estimates.

5.6 Conclusion

The present study found that higher dietary intakes of vitamin C were cross-sectionally associated with 0.6% higher VOS in men and up to 4.2% higher BUA in women. These differences in bone density between subjects with low and high vitamin C intakes may have important implications for fracture risk in the long term. Moreover, higher plasma vitamin C concentrations were a significant predictor of reduced fracture risk in men, with the greatest protection found at the spine. The present findings highlight the importance of fruits and vegetables in our diet, being the main sources of dietary vitamin C, and may suggest that as little as one or two additional portions of vitamin C-rich foods per day, such as citrus fruits, could have important implications for bone health. The present vitamin C investigations provide novel cross-sectional data as well as prospective data for vitamin C intake and status associations in a UK population of older men and women. Future studies should consider RCTs investigating the direct effects of vitamin C intake on indicators of bone health in humans. This has not been conducted previously and will be an important step in confirming the present observational findings as well as allowing for further understanding of the potential mechanisms involved.

CHAPTER 6

IRON AND BONE HEALTH

Cross-sectional and prospective investigations of iron intake and serum ferritin with heel ultrasound and fracture risk

6.1 Abstract

Iron plays a crucial cofactor role in the hydroxylation reactions within bone collagen fibres, thereby increasing overall collagen strength, as well as in the synthesis of vitamin D, an important mediator of calcium absorption. Previous epidemiological studies have shown positive associations between dietary iron intakes and bone density, and inverse associations with bone loss. However, studies investigating markers of iron status have had contradictory findings, possibly due to short follow-up and small sample sizes. To date, epidemiological evidence from large studies of men and women is limited, especially from British populations, and prospective data on long-term fracture risk is lacking. Therefore, this study aimed to explore i) potential cross-sectional associations between dietary iron intakes and serum ferritin concentrations as a marker of iron stores with measures of heel ultrasound and ii) potential prospective associations between iron intake and serum status with the risk of fractures in a sub-set of the 25,639 EPIC-Norfolk men and women aged 39-79 years at baseline. The results from the cross-sectional study showed that iron intake, but not serum ferritin, was significantly associated with 4.4% higher BUA in women only. The largest difference in mean dietary iron intake between the extreme quintile groups in women was 9 mg/d, and this is achievable through the usual diet, although particular attention should be paid to consuming a variety of iron-rich foods. In the prospective study, higher compared to the lowest iron intakes were significantly associated with 35% lower total fracture risk and up to 59% lower spine fracture risk in women only. Moreover, spine fracture risk was up to 70% lower in women with higher compared to the lowest serum ferritin concentrations. There were no associations between iron intake or serum ferritin with heel ultrasound and fracture risk in men in this cohort. The present study provides novel prospective data on long-term fracture risk with iron intake and status, and addresses a number of limitations of previous cross-sectional studies including providing data for British men and women and using a larger sample size. The present findings highlight the importance of an adequate iron intake and iron status in women. Future studies should conduct RCTs to investigate the effects of iron supplementation on changes in bone density and fracture risk, as this has not been conducted before.

6.2 Introduction

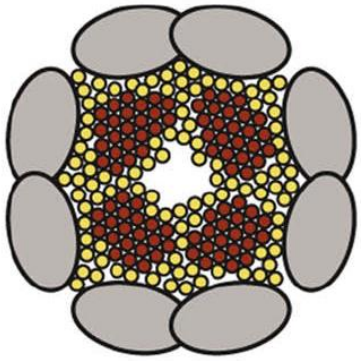
The first connections between iron and bone health were made in the late 1950's where bone changes were noted in subjects with chronic iron deficiency. Initially, x-ray-based reports were confined to alterations of the skull [352-354]; however, further investigations revealed more pronounced bone changes such as osteoporosis in the short and long bones [355]. It was proposed that these changes may reflect bone marrow hyperplasia initiated by chronic iron deficiency anaemia in conjunction with other dietary deficiencies [355]. Later investigations reported that iron is crucial for adequate bone collagen synthesis and maintenance, acting as a cofactor in the hydroxylation of proline and lysine residues within a collagen fibre [9], thus contributing to adequate formation of collagen cross-links and subsequent stronger collagen fibres. Collagen is an essential component of bone tissue with around 98% of the organic phase of bone being comprised of type I collagen [13]. Iron is also involved in converting vitamin D, an important mediator of calcium homeostasis [125], into its active form (1,25-dihydroxycholecalciferol) via acting as a cofactor to the reaction-specific enzyme 25-hydroxycholecalciferol 1-hydroxylase [172].

Iron is an essential mineral to humans and is one of the most abundant metals in the body [356]. The body has an efficient mechanism of recycling iron from degraded red blood cells, thus its daily nutritional requirements are relatively small (1.0-1.5 mg/d) [357]. Dietary iron may be derived from animal and plant sources with varying degrees of absorption and bioavailability in the human gut. Approximately 15-30% of mainly animal-derived ferrous iron (Fe^{2+}) and 3-15% of predominantly plant-based-derived ferric iron (Fe^{3+}) may be absorbed, predominantly depending on factors including the iron content and composition of the meal and an individual's iron status [358]. The absorption of Fe^{3+} may also be enhanced by the simultaneous presence of a reducing agent such as vitamin C in the gut [359]. The absorption of iron in general may also be inhibited by a number of factors including high intakes of other minerals and trace elements. For example, calcium consumed both as part of the diet (calcium-containing foods) and as supplements may be one of the most inhibiting compounds for iron [360-363], possibly by interfering with iron transport within intestinal mucosal cells [364]. In contrast, calcium has not been shown to interfere with iron stores [360, 365, 366].

In the UK, the main sources of iron intake in adults are iron-fortified cereals and cereal products (45%), such as breakfast cereals and white bread [367]. Other contributors to dietary iron intake include meat and meat products (15-19%) and vegetables (16-19%). The intake of iron from both foods and supplements in the UK population is estimated to be 10-15 mg/d in men and 8-13 mg/d in women [367]. Men have higher total intakes of iron than women; however, when correcting for total energy intake both sexes were shown to have a similar iron density of the diet of around 0.3 mg/1000kcal/day [367]. In the UK, the RNI and LRNI for adults are 8.7 mg/d

and 4.7 mg/d, respectively, for both men and post-menopausal women, and at 14.8 mg/d and 8 mg/d for women of reproductive age [225]. A comparison with daily intakes showed that women of reproductive age only met approximately 66-87% of the RNI, whereas recommendations were met by older women and men [367]. Persistent low intakes of iron may eventually lead to iron deficiency anaemia which is defined as a blood haemoglobin levels below 130 g/l in men and 120 g/l in women aged 15 years and over [368, 369]. Anaemia is suggested to be the most prevalent nutrient deficiency worldwide, affecting approximately 1.6 billion people, particularly women of reproductive age [368], and it has been associated with reduced cognitive and physical development in children, reduced physical performance such as work productivity in adults, an increased risk of maternal and child mortality, susceptibility to falling and frailty [368, 370].

Figure 6.1: A proposed schematic cross-section of ferritin in the liver.



Ferritin consists of protein shell subunits (grey lobes) which surround a central cavity. The latter holds loosely packed ferric ions (yellow circles) and tightly packed ferric ions (red circles) forming eight ferrihydrate crystal structures (four of which are shown here). From Pan et al. (2009) [371].

Most of the iron stored in the body is bound to ferritin, a protein containing up to 4000 iron atoms in its centre **(Figure 6.1)** [371, 372]. Ferritin is primarily present in the liver, spleen, bone marrow and skeletal muscles and only very small amounts of the molecule are secreted into the blood. Ferritin measured in blood is the most sensitive indicator of iron stores, for example a plasma ferritin concentration of 1 µg/l may reflect iron stores of 140-180 µmol/l (8-10 mg) [373]. Ferritin is also a useful indicator of long-term high or inadequate dietary iron intake [374] because it accounts for the varying bioavailability of iron to the body, which in turn is dependent on factors such as the source of iron intake and current body stores [374]. The normal range of serum ferritin varies by sex, with men having higher concentrations than women, and by age [357, 372]. In men, serum ferritin concentrations peak between the ages of 30 and 39 years and thereafter remain constant until the age of 70 years. In women, levels are much lower during the reproductive ages, but start rising post menopause [375]. Serum ferritin levels are considered normal at ranges between 20-300 µg/l in men and 15-150 µg/l in women [376]. Lower concentrations indicate depleted iron stores [369, 376]. In 1998, the National Diet and Nutrition

Survey for people aged 65 years and over reported that 7% and 9% of British men and women, respectively, had depleted serum ferritin concentrations [223]. Levels above the normal sex-specific range were reported for 6% of men and 11% of women. Elevated levels may be a result of inflammation and infection or, in the absence of these, are indicative of iron overload (haemochromatosis).

Interestingly, correlations between serum ferritin and dietary iron intakes are generally very low, with significant but weak correlation coefficients of r=0.14-0.15 ($P \leq 0.03$) previously reported [223, 377]. In contrast, a number of studies failed to show a significant association between dietary iron intakes and serum ferritin concentrations in different population groups including adolescents and adult men and women [378-382]. Potential explanations for these findings may be related to a number of factors, including i) variations in the biological availability of iron from foods which is mediated amongst other factors by the degree of intestinal absorption, the composition of meals and individual iron requirements, ii) physiological iron losses such as menstruation and other iron losses, for example, blood donation which are independent of dietary iron intake, iii) the use of iron supplements, and iv) errors associated with the reporting of iron intake or measuring ferritin concentrations in blood [379].

To date, the evidence for a potential protective role of iron intake and body iron stores for developing osteoporosis and fractures is scarce, despite a number of suggested underlying mechanisms in bone health including the production and maintenance of bone collagen and vitamin D synthesis [9, 172].

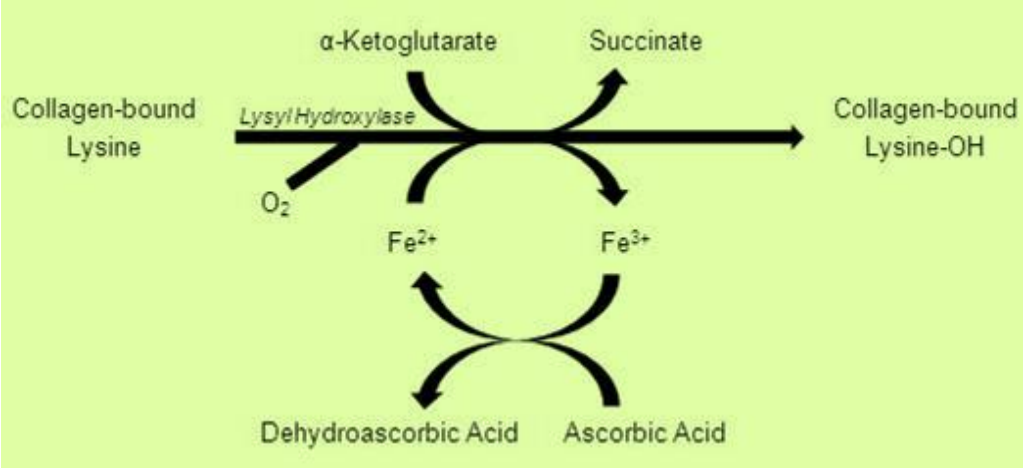
6.2.1 The potential role of iron in bone health

6.2.1.1 Bone collagen synthesis

It is well established that iron, alongside a reducing agent, oxygen and α-ketoglutarate, is an essential activator of enzymes involved in the hydroxylation of prolyl and lysyl residues within collagen fibres **(Figure 6.2)** [8, 10]. Residues of lysine and proline, an essential and non-essential amino acid respectively, get hydroxylised to form hydroxylysine and hydroxyproline. This reaction is a crucial step in bone collagen synthesis, as the subsequent formation of covalent bonds between adjacent collagen fibres leads to stronger collagen cross-links, thus increasing overall collagen strength [136]. Mechanistic studies of the relative importance of iron in the hydroxylation reaction have shown that there was an absolute dependence of iron for prolyl enzyme activity, as no hydroxylation took place in its absence [322]. In concordance with this, it has been hypothesised that in iron deficiency, the lesser amount of iron available to the hydroxylation enzymes may lead to decreased cross-linking activity and subsequent weakened collagen fibres [322]. As both bone and cartilage contain a structurally stable network of collagen,

impaired collagen synthesis resulting from inadequate iron intake may potentially be a risk factor for the development of osteoporosis and associated fractures.

Figure 6.2: The hydroxylation of lysine in collagen fibres.

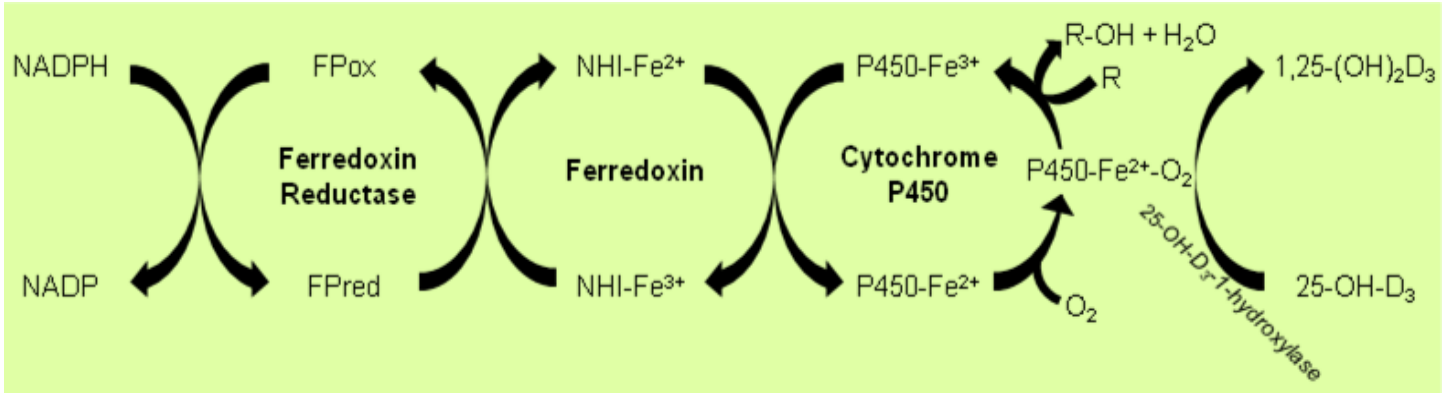


Adapted from Medeiros & Wildman (2011) [(324)].

6.2.1.2 Vitamin D synthesis

Another important role of iron in maintaining bone health is its involvement in vitamin D synthesis, thereby affecting calcium absorption. Calcium is one of the fundamental bone-forming compounds and vitamin D is an important mediator of calcium homeostasis by increasing calcium absorption efficiency [(125)]. During vitamin D synthesis, in response to low wavelength-dependent UV light exposure of the skin, iron is involved in converting 25-hydroxyvitamin D into its active form 1,25-dihydroxycholecalciferol in the kidneys **(Figure 6.3)**. Iron in the form of ferredoxin, alongside a ferredoxin reductase and a cytochrome P450, acts as a cofactor to the reaction-specific enzyme 25-hydroxycholecalciferol-1-hydroxylase [(172, 383)]. It has previously been hypothesised that in iron deficiency the availability of iron to this enzyme may be limited, and this may lead to decreased vitamin D synthesis and subsequently lower intestinal calcium absorption [(177)]. Thus, insufficient dietary iron intake may also play a role in osteoporosis and fracture development by potentially lowering the mineralisation of bone tissue. Recently, a cross-sectional study of 554 US men and women showed that the prevalence of anaemia was significantly higher in vitamin D deficient subjects compared to those with normal vitamin D levels (49% *vs.* 36%, $P<0.01$) [(384)]. Moreover, participants with vitamin D deficiency had a significant OR for anaemia of 1.9 (95%CI 1.3-2.7). However, the potential relationship between iron deficiency and vitamin D deficiency remains to be investigated further in relation to bone health.

Figure 6.3: The role of iron in vitamin D synthesis.



Adapted from DeLuca (1976) [(172)] and Jones et al. (1998) [(383)].

6.2.1.3 Both iron deficiency and iron overload may have unfavourable effects on bone health

There is evidence that iron at both deficient and very high levels may exert negative effects on bone and potentially promote the development of osteoporosis. In iron deficiency, as discussed above, the limited amount of iron available may compromise adequate collagen formation and vitamin D synthesis [177, 322]. Recently, different aspects of bone turnover have also been shown to be mediated. For example, whereas low concentrations of iron (induced by 5 µmol/l of deferoxamine, an iron chelating agent) promoted osteoblast activity in human osteoblastic cells, very low concentrations (induced by 10-20 µmol/l of deferoxamine) had an inhibitory effect [385]. Moreover, low concentrations of iron inhibited osteoblastogenesis *in vitro* [386].

The notion that iron at high concentrations may contribute to the development of osteoporosis is evident in patients with hereditary iron overload (haemochromatosis) or impaired iron metabolism such as in sickle cell anaemia, both characterised by an accumulation of iron in the body [387, 388]. For example, in observational studies, osteoporosis (T-score of -2.5 SD) and osteopenia (T-score of -1 SD) were present in 25-34% and 41-79% of haemochromatosis patients, respectively [387, 389, 390]. In recent years, numerous animal models (including mouse, rat and pig) showed that experimental or genetically-induced iron overload negatively impacted on bone turnover. For example, an excessive iron status was associated with an increase in the number of osteoclasts and upregulated osteoclast activity [391-393]. Moreover, iron overload was shown to inhibit osteoblast gene expression and osteoblast activity in a concentration-dependent manner [385, 394-396]. An increase in the concentration of intracellular reactive oxygen species (ROS) was also reported *in vitro* [392, 395, 397]. It was subsequently suggested that oxidative stress in iron overload may decrease bone formation by inhibiting osteoblast activity [395], and increase bone resorption via upregulating RANKL-induced osteoclastogenesis as well as the expression of the pro-inflammatory cytokines TNF-α and IL-6 [392, 397]. This is in agreement with a recent mouse model, where levels of CTx (a marker of bone resorption) and TNF-α were approximately 50% higher in the iron overload mice compared to the controls [398]. The reported changes in bone cell synthesis, bone cell activity and reactive oxygen species expression in these animal and cell studies may suggest that iron overload potentially causes an increase in bone resorption and a simultaneous decrease in bone formation. Such an imbalance in bone turnover is known to be linked with increased bone loss and subsequently compromised bone strength in the long-term. In concordance with this, a recent study in mice showed that iron overload was associated with a decrease in bone load-bearing capacity (a measure of bone elasticity and its ability to withstand bending stress), and subsequently a higher risk for developing fractures [398].

6.2.2 Associations between iron and bone health in previous studies

Despite suggestions of a potential mechanistic role for dietary iron in bone health, this has not received much attention, possibly due to the simultaneous emerging interests in calcium and vitamin D for bone health. However, there is evidence from animal studies reporting lower BMD and BMC, reduced mechanical strength and increased porosity in iron deficient and severely iron deficient rats and rats on iron-restricted diets [173-176, 399, 400]. The rate of bone turnover was also mediated [173, 174, 177, 400], predominantly evident by decreased bone formation and increased bone resorption. Moreover, the degree of bone mineralisation was reduced as evident by a decrease in calcium, phosphorus and magnesium content of bone despite adequate dietary intakes of these nutrients [177, 401]. *In vitro* studies partly supported previous *in vivo* findings, showing that iron deficiency, induced by an iron chelator, resulted in up to 70% less mineralised surface area ($P<0.05$) [386, 399], possibly due to inhibited differentiation into osteoblasts [386]. To date, there is limited evidence from epidemiological studies for a potential role of iron in reducing osteoporosis risk, and from RCTs investigating the effects of iron supplementation on surrogate markers of bone health. The detailed findings of the literature review are discussed in the following sections, and the evidence is presented in hierarchical order of decreasing ability to infer causality.

6.2.2.1 Intervention studies and randomised controlled trials

Despite evidence from many animal studies, evidence for a potential role of iron in osteoporosis and fracture prevention from human RCTs is limited. RCTs are considered to be the gold standard for inferring causality. They can determine which factors influence disease, limiting both selection biases and confounding. To our knowledge, only three double-blind RCTs have recently been published by a Spanish research group, and a summary table of these studies can be found in **Appendix 4, Table A4.1**. The trials measured the effects of iron supplementation on the bone formation markers BSALP or PINP and the bone resorption marker NTx in young Spanish women in different settings. In the largest of the three RCTs, 165 women aged 18-35 years received either no supplementation (control group: iron sufficient) or 15 mg/d of iron with or without vitamin D (5 µg/d) using fortified skimmed milk (treatment groups: iron deficient) for four months [180]. The supplementation with iron alone had no effect on markers of bone turnover; however, the results may have been affected by the chosen supplementation medium. Calcium and casein, which are naturally present in milk, are strong inhibitors of iron absorption [361, 362]. In fact, the authors reported that there was no improvement in iron status in those women receiving the iron-fortified milk, suggesting that the treatment was ineffective. The additional fortification of the milk with vitamin D resulted in a significant reduction in bone resorption over the four months, although this is most likely not related to iron as beneficial

effects of vitamin D for bone health have previously been shown [121, 402]. Thus, definitive conclusions regarding the effects of iron on markers of bone turnover may not be drawn from this trial. The smallest RCT by the same research group used fruit juice as the supplementation medium [179]. In this study, 41 iron deficient women also aged 18-35 years were given 500ml of either a placebo fruit juice or a fruit juice fortified with 18mg/d of iron pyrophosphate for four months. The results showed that markers of bone turnover (BSALP and NTx) were not affected by the supplementation, despite the improved iron status of the treatment group. The non-significant findings may be explained by the time period the RCT was undertaken, which was for four months during the winter months. The authors reported that vitamin D status was significantly lower in all women at the end of the study, with 75% of women having 25-hydroxyvitamin D levels below 45 nmol/l (vitamin D deficiency). This may reflect the insufficient UV light exposure and subsequent lack of vitamin D synthesis during the winter months in populations living at latitudes higher than 40° [403]. Low levels of circulating 25 hydroxyvitamin D have previously been associated with low BMD [404], and these mechanisms may have overpowered the potentially beneficial effects of iron on bone markers in this population. Finally, the third RCT (*n*=73) compared bone turnover markers between anaemic women (mean age: 35±5 years) and healthy women (mean age: 28±3 years) [178]. The results showed that bone resorption at baseline, measured from NTx levels, was significantly higher in anaemic women compared to healthy controls (37.8±16.5 *vs.* 21.9±8.4 nmol bone collagen equivalents (BCE)/mmol creatinine, *P*<0.001), but there were no differences in the rate of bone formation between the two groups. The RCT also investigated the effects of recovering from anaemia via the supplementation with ferrous sulphate tablets (iron: 80-160 mg/d) for 2-4 months on markers of bone turnover. The results showed that, in women who recovered from anaemia, PINP levels decreased significantly from 41.2±17.5 to 32.6±14.5 ng/ml (*P*<0.001) and NTx levels from 40.0±17.2 to 31.0±9.9 nmol BCE / mmol creatinine (*P*<0.05), indicating that the recovery from anaemia is associated with a decrease in the rate of both bone formation and bone resorption. No such changes were observed in those women who remained anaemic following the iron treatment. Moreover, there were no significant differences in markers of bone turnover between the two groups at both baseline and end of treatment.

To date, there is data from only three RCTs, all originating from one Spanish research group, which investigated the effects of iron supplementation on the bone formation markers BSALP or PINP and the bone resorption marker NTx in young Spanish women in different settings. The results were predominantly non-significant; however, issues regarding the small sample size (*n*=41-165), the supplementation medium (milk) and the period of study (winter months) were identified, and those may have affected the study results. Moreover, no RCTs have included men, and RCTs which use changes in BMD as an outcome measure have not been conducted yet. Thus, larger RCTs are needed which run for several years and which investigate

the effects of iron supplementation on indicators of bone health including changes in BMD in larger populations of men and women.

6.2.2.2 Prospective studies

Prospective cohort studies are a preferred methodology of investigating the aetiology of a disease with less recall bias than case-control studies as the exposure is measured prior to the disease occurring. In these studies, selection bias is also limited because the cases and controls are drawn from the same population. To the best of our knowledge, there are only three prospective studies on iron and bone health, and those are summarised in a table in **Appendix 4, Table A4.2**. All studies explored potential associations between dietary iron intake or status and changes in BMD [185, 186, 405], and one additionally investigated incident vertebral fractures [185]. One study was undertaken in 228 US postmenopausal women aged 40-65 years with a mean iron intake of 15±5 mg/d [405]. Iron intake from foods was determined from eight randomly selected days of diet records completed for two to three weeks at three different time points over the course of one year. The study showed that iron intake was significantly and positively associated with 1-year change in BMD at the trochanter (β 0.041±0.017 g/cm^2, *P*=0.015) and the Ward's triangle (β 0.055±0.026 g/cm^2, *P*=0.037). Moreover, iron intake accounted for around 3-9% of the variance in BMD change. The study also investigated other BMD sites including the femoral neck, lumbar spine and total body, but those were not found to be associated with BMD change. Potential explanations for this may be the small sample size of less than 230 women which may have limited the statistical power of the study to detect significant associations. Another limitation may have been the short follow-up period of only one year. Bone turnover is generally a very slow process with one remodeling cycle at any one site taking approximately between three and six months [19, 20], thus any changes in BMD or lack of thereof may have been more pronounced after several years of follow-up. The second prospective study on iron and bone health had a longer follow-up time of 3.5-5 years, but investigations were undertaken in a much smaller study population of 32 British postmenopausal women aged 46-55 years [186]. The dietary iron intake in these women was 12 mg/d (*SD not reported*) and was estimated from 7-13 individual food diaries which had been completed for either three or seven days. When iron intake was adjusted for energy intake, it was significantly correlated with less spinal BMD loss over the 3.5-5-year follow-up period (r=0.42, *P*=0.02). Moreover, higher intakes of iron were also significantly associated with less BMD loss at the spine following the adjustment for energy intake and BMI (β 0.141±*SD not reported* g/cm/year, *P*<0.0001). Despite these positive findings, the study was limited by the very small sample size of only 32 women, as previously discussed. Moreover, the study did not adjust for covariates known to be associated with bone health including smoking and exercise. Furthermore, the measurement of spinal BMD was performed using dual photon absorptiometry (DPA) at baseline and some of the earlier time points;

however, DXA was used at later follow-up time points. Although this was adjusted for by the use of conversion factors, the use of different measuring equipment during the course of the study may still not have been as accurate as if the same method had been used throughout. The largest prospective study was undertaken in 1729 Korean men and women aged 56±8 years and had a follow-up time of three years [185]. The results showed potential detrimental effects of higher iron status for bone health. The annualised bone loss rate at different hip sites was significantly faster in quartile 4 compared to 1 of serum ferritin concentrations in both men (78-113%) and women (34-37%, $P \leq 0.023$); and this inverse trend was found to be significant across all quartiles (P-trend≤0.043). Moreover, the odds for morphological vertebral fracture risk were positively associated with ferritin in women but not in men (P-trend=0.023), with women in quartile 4 compared to those in quartile 1 having a significant five-fold higher fracture risk (OR 5.27, 95%CI 1.12-24.94). A limitation to the study was that women in the top quartile of serum ferritin tended to be older than those women in the lowest quartile (56.5±6.5 *vs.* 55.4±6.0, P=0.07), and increasing age has previously been associated with lower BMD [30, 31]. Thus, the detrimental effects of increasing age for bone health may have masked the associations with serum ferritin in this study. Moreover, the follow-up of three years was very short for exploring the association between iron status and fracture risk.

To date, there is some evidence for a potentially beneficial effect of iron intake for preserving bone loss. However, current findings are limited to only two prospective studies which were undertaken in very small populations, and one study had a very short follow-up period. Moreover, both studies were performed in women and no prospective studies to date have included men, despite osteoporosis becoming an increasingly greater health burden in both sexes [171]. Only one prospective study has investigated associations with measures of stored iron in the body and found potential harmful effects of higher serum ferritin for bone health in men and women and for fracture risk in women only. However, this study had a short follow-up of only three years and may have been biased by the known detrimental effects of age on bone health. To date, no prospective studies have investigated potential associations between iron intake or serum concentrations with long-term fracture risk in a large population of men and women.

6.2.2.3 Case-control studies

In case-control studies, specific exposures in people with and without a pre-defined condition are being compared in order to determine their potential as risk factors of the disease. Both exposure and disease outcome are pre-defined but the exposure measurement must precede the development of the condition. Hence, case subjects may report specific exposures inaccurately as a result of their experience of symptoms, resulting in both selection bias and recall bias. To the best of our knowledge, there are no case-control studies investigating the

potential relationship between dietary iron intakes or iron stores with the prevalence of osteoporosis and fractures up to this date.

6.2.2.4 Cross-sectional studies

In cross-sectional studies, it cannot be determined whether or not the exposure predated the disease, as investigations are limited to a specific point in time. However, these studies are still useful for understanding the prevalence of a disease in a defined population and for hypothesis generation. A summary table of previous cross-sectional studies investigating potential associations between iron and bone health can be found in **Appendix 4, Table A4.3**. To date, there is some evidence from cross-sectional studies for higher dietary iron intakes or iron status potentially being important for osteoporosis prevention. To our knowledge, four cross-sectional studies have previously investigated iron intake in relation to BMD, and those have had some significant findings. For example in two studies of 242-244 postmenopausal US women, higher iron intake was significantly associated with higher BMD at all investigated sites including the spine, femoral neck, femoral trochanter, Ward's triangle and total body (β 0.085-0.251 g/cm^2, $P \leq 0.01$) [181] and (β 0.214-0.426 g/cm^3, $P \leq 0.05$) [142]. Moreover, the highest compared to the lowest iron intake (*details of intakes not reported*) was also associated with 4-14% higher BMD at all sites ($P \leq 0.05$) [181]. Another cross-sectional study of 175 Swedish women aged 28-74 years also found significant associations between higher dietary iron intakes and BMD; however, the results were only significant in the univariate analyses (β 0.0069-0.011, $P \leq 0.02$) [406]. Potential explanations for this may relate to the slightly smaller sample size compared with the two US studies and the multivariate analysis not including important covariates such as dietary calcium intakes and the use of supplements. The smallest cross-sectional study investigating potential associations between iron intake and BMD was undertaken in 159 Australian pre-menopausal and postmenopausal women [407]. The study did not account for a number of covariates known to affect bone health including smoking, exercise and dietary calcium intakes which must be considered when interpreting the study outcomes. The results showed that iron intake was an independent predictor of BMD at the femoral neck alongside age and weight (R^2=0.25, P<0.001), but only in pre-menopausal not postmenopausal women. Iron intake in pre-menopausal women was also significantly positively correlated with femoral neck BMD (r=0.24, P<0.05) and bone mineral content of the forearm (r=0.26, P<0.05). Despite some evidence for a potential role of dietary iron intake in osteoporosis prevention, to date, there is data from only four cross-sectional studies. Those were undertaken in women only and had very small sample sizes (n=159-244) which may have limited their power to detect all potential associations. Moreover, the studies used different means of assessing dietary iron intake, including a 3dDD [181] and a weighed 4dDD [407], or a combination of methods such as an FFQ and four 7dDDs [406], and an FFQ and eight 24hRs [142]. To our knowledge, there is also no cross-sectional data from British

populations. Thus, further studies are needed which will investigate potential iron-bone associations in both men and women using populations of larger sample sizes, as this has not been done before.

Associations between iron status and BMD have previously been investigated in five cross-sectional studies. The smallest study was undertaken in 455 Italian postmenopausal women and showed that there was a significant inverse correlation between serum transferrin and BMD at the spine (r=-0.2, *P*=0.015) and the hip (r=-0.34, *P*<0.001), although no such associations were found with serum iron or ferritin concentrations [(183)]. Another cross-sectional study also investigated serum iron concentrations in 728 Turkish women (mean age: 57±6 years) [(408)]. They found that in women aged 45-59 years, serum iron concentrations were significantly lower in those with osteoporosis at the femoral neck (101.1±45.0 *vs.* 90.7±43.1 μg/dl, *P*=0.030) and total hip (101.0±44.7 *vs.* 86.8±43.4 μg/dl, *P*=0.012) compared to women without osteoporosis, although no such observations were made in older women aged 60-79 years. Moreover, there were no differences in the risk of osteoporosis at multiple sites between women with low and normal serum iron concentrations in either age group (OR 1.0, 95%CI 0.6-1.9, *P*≥0.33). Another cross-sectional study investigated potential associations between the prevalence of anaemia with trabecular and cortical BMD and bone area in 950 Italian men and women aged 65-102 years [(184)]. The results showed that women with anaemia compared to those without had significantly lower trabecular BMD (179±76 *vs.* 199±59 mg/cm^3, *P*=0.02) and cortical BMD (935±96 *vs.* 988±71 mg/cm^3, *P*<0.001); whereas men with anaemia had significantly lower cortical bone density only (993±87 *vs.* 1019±62 mg/cm^3, *P*=0.01). Moreover, in both men and women, every SD increase in bone density at multiple sites was significantly associated with higher haemoglobin levels (β 0.076-0.112, *P*≤0.04), and in women only, with a lower prevalence of anaemia (β -0.335-(-)0.428, *P*≤0.04). A larger cross-sectional study in 2943 South Korean men and women aged 65 years and over investigated associations between serum ferritin concentrations and BMD at the hip and spine [(182)]. The results did not show any significant associations in women; however in men, higher serum ferritin levels were significantly associated with higher BMD at all investigated sites (β 0.008-0.018 ± 0.004-0.005 (SE), *P*≤0.049). Moreover in men, BMD at all sites increased and the prevalence of osteoporosis decreased significantly across tertiles of serum ferritin (*P*≤0.022). The largest cross-sectional study investigating potential associations between iron status and BMD at the spine and hip was a recent study in 5148 Korean men and women aged 10-95 years which reported potential detrimental effects of higher iron status for bone health [(409)]. The results were sex-specific and showed that serum ferritin concentrations were significantly inversely associated with all BMD sites in women aged ≥45 years and with spine BMD in 25-44 year old women only (β -0.012-(-)0.039 ± 0.005-0.007 (SE), *P*≤0.041). Further investigations in women (≥45 years) showed that spine BMD was significantly lower in women in the two upper quartiles compared to those

women in the lowest quartile of ferritin (3.2-3.4%, *P*<0.05). Moreover, the odds for osteoporosis were significantly higher in women (≥50 years) in the two upper compared to the lower quartile of ferritin (quartile 3vs1: OR 1.45, 1.02-2.05; quartile 4vs1: OR 1.55, 1.09-2.23). Similarly, the odds for self-reported fractures were also significantly higher in women in quartile 4 compared to those in quartile 1 (OR 1.52, 1.02-2.27).

Despite all published cross-sectional studies showing some significant associations between iron status and bone health, the results were contradictory as both potentially beneficial and detrimental associations were found. However, the data are limited to five studies which used different exposure assessments and outcome measures to investigate the role of iron status in bone health. Moreover, only two studies included a large population of more than 2000 participants and those were undertaken in Korea, whereas no cross-sectional studies have been undertaken in British populations. Future cross-sectional studies need to investigate the potential relationship between iron status and bone health in a large population of men and women, particularly of British origin.

6.2.2.5 Summary of previously published studies

To date, a significant proportion of evidence for a potential role of iron sufficiency in bone health has come from animal studies, but data in humans are limited. To the best of my knowledge, there is data from only three RCTs, three prospective studies and nine cross-sectional studies, but no case-control studies. The results have been inconsistent, possibly due to the small sample sizes. For example, RCT evidence has shown that the rate of bone resorption was significantly higher in anaemic women compared to controls; whereas other RCTs did not find any evidence that iron supplementation may be beneficial to bone health. However, the results may have been affected by the small sample sizes of the RCTs, the pre-existing iron status of the population, the supplementation medium and the period of study; and all published RCTs were conducted in only women. In prospective studies, higher dietary iron intake was significantly associated with less BMD loss over time, but these investigations were undertaken in women only. Issues regarding small sample sizes, a short follow-up period and changes in BMD measuring equipment during the course of the study may have affected the study outcomes. Furthermore, data from prospective studies investigating iron stores rather than dietary iron intakes is available from only one study which found potentially harmful effects of higher iron stores for bone health in men and women and for fracture risk in women only. However, issues regarding a short follow-up and masking detrimental effects of age have been identified. To date, data from prospective studies investigating long-term fracture risk in a large population of men and women is lacking. Some but not all cross-sectional studies have found significant associations between higher dietary iron intakes and higher BMD at multiple sites, although all studies had small sample sizes and were conducted in women only. Cross-sectional associations

between iron status and BMD have also previously been reported, although both positive and negative relationships were found. However, only two of those studies were undertaken in large populations of more than 2000 participants, and no studies have been undertaken in British populations.

In conclusion, as a range of potential underlying mechanisms for iron and bone have previously been suggested, and iron deficiency anaemia is considered to be the most prevalent global nutrient deficiency, more epidemiological evidence for a potential association between iron intake and status with indicators of bone health is needed. Evidence is particularly limited from epidemiological studies of large populations of men and women. For prospective studies, investigations should be conducted for both dietary iron intake and iron status; and outcome measures should include both BMD and fracture risk after many years of follow-up. Future cross-sectional studies should investigate iron intake and status in the same population as this has not previously been done before. Moreover, there is no evidence for a potential role of iron in bone health in British populations, thus data from UK cohorts is needed.

6.2.3 Chapter aims and objectives

In order to address some of these limitations, this chapter aimed to:

i) Investigate potential cross-sectional associations between dietary iron intake as well as serum ferritin concentrations with the heel ultrasound parameters BUA and VOS.

ii) Examine potential prospective associations between dietary iron intake and serum ferritin concentrations with the risk of fracture at the hip, spine and wrist in a British population of men and women aged between 39 and 79 years at baseline.

This study will provide novel investigations of potential associations between dietary iron intake and serum ferritin with the long-term risk for fractures. Moreover, the findings will also provide more evidence in a British population, particularly in men where data is limited, and will use a larger sample size than most previous studies. It was hypothesised that dietary intakes of iron and serum ferritin are positively associated with measures of bone density and inversely associated with the risk of fracture.

6.3 Methods

Two types of analyses were undertaken on a randomly selected sample of men and women of the EPIC-Norfolk prospective cohort study, as discussed in detail in Chapter 2 (page 40). Briefly, the cross-sectional study of heel ultrasound was based on a random sub-cohort of 4000 participants who had attended the first health check, and the prospective investigations of fracture risk were based on a case-cohort design using the same subset of 4000 participants and a set of 1502 participants who had experienced a fracture up to 31st March 2009. For both types of studies, analyses using iron as the predictor variable were performed using quintiles and were undertaken for i) iron intake from the diet and ii) serum ferritin concentrations. Firstly, multiple regressions determined the cross-sectional relation of quintiles of dietary iron intake from foods and quintiles of serum ferritin concentrations with BUA and VOS. Both BUA and VOS are measures of heel ultrasound, but BUA is an indicator of the structural organisation of bone, whereas VOS determines bone stiffness [63]. Secondly, the differences in crude total fracture incidence over the median 12.6-year follow-up between the quintile groups was evaluated by computing Kaplan-Meier survival curves alongside log-rank tests of equality. Then, potential prospective associations between quintiles of dietary iron intake and quintiles of serum ferritin concentrations with fracture risk at the hip, spine and wrist were investigated using Prentice-weighted Cox proportional hazard ratios [221]. For both the cross-sectional and the prospective studies, potential associations between the extreme quintiles of iron intake or serum ferritin concentrations referent to the lowest quintile were investigated. As previously discussed, all analyses were stratified by sex and adjusted for relevant confounders using an unadjusted and two multivariate models (Chapter 2, page 49). The final model included age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. The procedures for dealing with missing data and the number of exclusions in each study are discussed in detail in Chapter 3 (pages 55-57).

6.4 Results

6.4.1 Cohort descriptives

6.4.1.1 Descriptive statistics stratified by quintiles of dietary iron intake

Characteristics of the 4711 EPIC-Norfolk participants stratified by quintiles of dietary iron intake are presented in **Table 6.1**. In the 1957 men, mean±SD iron intakes for the quintile groups were as follows: Q1 8.6±1.3 mg/d, Q2 11.0±0.5 mg/d, Q3 12.8±0.6 mg/d, Q4 14.8±0.7 mg/d and Q5 19.3±3.9 mg/d. Mean iron intakes for each quintile were slightly lower in the 2754 women: Q1 6.9±1.0 mg/d, Q2 9.0±0.4 mg/d, Q3 10.4±0.4 mg/d, Q4 12.0±0.6 mg/d and Q5 16.1±2.8 mg/d. There were no differences in family history of osteoporosis, steroid use and calcium supplement use between the quintile groups. However, those with the lowest compared to higher iron intakes were least likely to use vitamin D supplements ($P \leq 0.007$). Moreover, there were significant decreases in age ($P<0.001$) across quintiles of iron intake. For example, men were 61.9±9.5 and 58.1±9.4 years old in quintiles 1 and 5 respectively, and women were 62.1±9.7 and 58.2±9.5 years. A similar decrease across the quintiles was also found for BMI ($P \leq 0.041$). Participants with higher compared to the lowest iron intakes were also more likely to be non-smokers and more physically active ($P<0.001$). Serum ferritin levels increased significantly with higher iron intakes in men ($P=0.024$), but a significant decrease was found in women ($P=0.014$).

6.4.1.2 Descriptive statistics stratified by quintiles of serum ferritin

Following the exclusion of those with missing information for serum ferritin ($n=1506$, 32%), characteristics of the 3205 participants stratified by quintiles of serum ferritin are presented in **Table 6.2**. In the 1385 men, mean±SD serum ferritin for the quintile groups were as follows: Q1 30.2±11.4 ng/ml, Q2 62.6±8.6 ng/ml, Q3 94.3±11.2 ng/ml, Q4 137.9±15.7 ng/ml and Q5 253.4±74.5 ng/ml. Mean serum ferritin for each quintile were lower in the 1820 women compared to men: Q1 16.5±5.1 ng/ml, Q2 33.3±4.8 ng/ml, Q3 51.0±5.7 ng/ml, Q4 75.7±9.3 ng/ml and Q5 147.4±56.4 ng/ml. There were no significant differences in smoking, physical activity, family history of osteoporosis, steroid medication and the use of calcium or vitamin D supplements between the quintile groups. Moreover, in women, HRT did not differ between the quintile groups. However, women with the highest serum ferritin concentrations were more likely to be post-menopausal compared to those with lower intakes ($P<0.001$). In line with this, there was a significant increase in age across quintiles of serum ferritin in women ($P<0.001$). For example, women were 56.4±9.7 and 63.0±8.0 years old in quintiles 1 and 5, respectively. In contrast, age significantly decreased in men ($P=0.003$), with men being 60.9±9.2 and 59.0±8.9 years old in quintiles 1 and 5, respectively. In both sexes, BMI increased significantly ($P<0.001$). In men, dietary iron intake increased significantly in men ($P=0.006$), but decreased in women ($P=0.042$).

Table 6.1: Baseline characteristics of the 1957 men and 2754 women of the EPIC-Norfolk case-cohort stratified by quintiles of dietary iron intake.

	Men											Women										
Dietary iron intake (mg/d)	**Quintile 1 3.4 – 10.1**		**Quintile 2 10.2 – 11.9**		**Quintile 3 12.0 – 13.7**		**Quintile 4 13.8 – 16.1**		**Quintile 5 16.2 - 42.2**			**Quintile 1 1.9 – 8.2**		**Quintile 2 8.3 – 9.6**		**Quintile 3 9.7 – 11.0**		**Quintile 4 11.1 – 13.1**		**Quintile 5 13.2 – 29.7**		
	n = 392		***n = 391***		***n = 392***		***n = 391***		***n = 391***		***P*-trend**	***n = 551***		***n = 551***		***n = 551***		***n = 551***		***n = 550***		***P*-trend**
Mean (SD)																						
Age (years)	61.9	(9.5)	60.5	(9.5)	60.1	(9.5)	57.9	(9.3)	58.1	(9.4)	*P*<0.001	62.1	(9.7)	59.9	(9.3)	59.3	(9.5)	59.4	(9.2)	58.2	(9.5)	*P*<0.001
BMI (kg/m^2)	26.8	(3.6)	26.5	(3.1)	26.6	(3.2)	26.2	(3.4)	26.4	(3.3)	*P*=0.041	26.8	(4.7)	26.4	(4.3)	26.0	(4.1)	25.9	(4.2)	25.7	(4.2)	*P*<0.001
Serum ferritin (ng/ml)†	112.0	(88.8)	110.5	(80.5)	111.7	(77.4)	117.8	(82.4)	126.4	(94.6)	*P*=0.024	70.6	(55.3)	65.5	(52.2)	63.0	(51.4)	63.7	(56.7)	60.9	(46.7)	*P*=0.014
n (%)																						
Menopausal Status																						*P*<0.001
Pre-mp	-	-	-	-	-	-	-	-	-	-		63	(11.4)	76	(13.8)	87	(15.8)	90	(16.3)	98	(17.8)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		17	(3.1)	18	(3.3)	29	(5.3)	18	(3.3)	45	(8.2)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		76	(13.8)	95	(17.2)	94	(17.0)	82	(14.9)	101	(18.4)	
Post-mp	-	-	-	-	-	-	-	-	-	-		395	(71.7)	362	(65.7)	341	(61.9)	361	(65.5)	306	(55.6)	
HRT																						*P*=0.033
Current User	-	-	-	-	-	-	-	-	-	-		71	(12.9)	96	(17.4)	91	(16.5)	111	(20.2)	103	(18.7)	
Former User	-	-	-	-	-	-	-	-	-	-		65	(11.8)	59	(10.7)	80	(14.5)	58	(10.5)	62	(11.3)	
Never Used	-	-	-	-	-	-	-	-	-	-		415	(75.3)	396	(71.9)	380	(69.0)	382	(69.3)	385	(70.0)	
Smoking											*P*<0.001											*P*<0.001
Current smoker	80	(20.4)	48	(12.3)	40	(10.2)	39	(10.0)	31	(7.9)		100	(18.2)	73	(13.3)	70	(12.7)	58	(10.5)	42	(7.6)	
Former smoker	223	(56.9)	207	(52.9)	215	(54.9)	216	(55.2)	220	(56.3)		178	(32.3)	167	(30.3)	174	(31.6)	167	(30.3)	203	(36.9)	
Never smoked	89	(22.7)	136	(34.8)	137	(34.9)	136	(34.8)	140	(35.8)		273	(49.5)	311	(56.4)	307	(55.7)	326	(59.2)	305	(55.5)	
Physical activity											*P*<0.001											*P*<0.001
Inactive	165	(42.1)	132	(33.8)	124	(31.6)	95	(24.3)	98	(25.1)		238	(43.2)	183	(33.2)	173	(31.4)	171	(31.0)	142	(25.8)	
Mod. inactive	87	(22.2)	93	(23.8)	104	(26.5)	94	(24.0)	93	(23.8)		159	(28.8)	187	(33.9)	183	(33.2)	165	(30.0)	183	(33.3)	
Mod. active	74	(18.9)	94	(24.0)	77	(19.7)	93	(23.8)	98	(25.1)		93	(16.9)	98	(17.8)	128	(23.2)	123	(22.3)	135	(24.5)	
Active	66	(16.8)	72	(18.4)	87	(22.2)	109	(27.9)	102	(26.0)		61	(11.1)	83	(15.1)	67	(12.2)	92	(16.7)	90	(16.4)	
Family history of OP	10	(2.6)	10	(2.6)	13	(3.3)	13	(3.3)	12	(3.1)	*P*=0.94	32	(5.8)	26	(4.7)	30	(5.4)	30	(5.4)	36	(6.7)	*P*=0.77
Steroids	21	(5.4)	9	(2.3)	13	(3.3)	12	(3.1)	13	(3.3)	*P*=0.20	27	(4.9)	26	(4.7)	22	(4.0)	21	(3.8)	18	(3.3)	*P*=0.65
Calcium supp.	4	(1.0)	2	(0.5)	8	(2.0)	5	(1.3)	6	(1.5)	*P*=0.40	19	(3.5)	34	(6.2)	31	(5.6)	32	(5.8)	39	(7.1)	*P*=0.11
Vitamin D supp.	70	(17.9)	72	(18.4)	106	(27.0)	96	(24.6)	86	(22.0)	*P*=0.007	134	(24.3)	171	(31.0)	179	(32.5)	184	(66.6)	207	(37.6)	*P*<0.001

Values are means (standard deviations) or numbers (frequencies).

† Serum ferritin levels were available for 1385 men and 1819 women.

Table 6.2: Baseline characteristics of the 1385 men and 1820 women of the EPIC-Norfolk case-cohort stratified by quintiles of serum ferritin.

	Men											Women										
Serum ferritin levels (ng/ml)	**Quintile 1 8.5 – 47.8**		**Quintile 2 47.9 – 76.0**		**Quintile 3 76.1 – 113.2**		**Quintile 4 113.3 – 169.2**		**Quintile 5 169.3 – 447.2**		***P*-trend**	**Quintile 1 8.0 – 25.1**		**Quintile 2 25.2 – 41.7**		**Quintile 3 41.8 – 61.3**		**Quintile 4 61.4 – 94.4**		**Quintile 5 94.5 – 442.9**		***P*-trend**
	n = 277		***n = 277***		***n = 277***		***n = 277***		***n = 277***			***n = 367***		***n = 362***		***n = 366***		***n = 361***		***n = 364***		
Mean (SD)																						
Age (years)	60.9	(9.2)	60.3	(9.7)	58.6	(9.5)	58.9	(9.2)	59.0	(8.9)	*P*=0.003	56.4	(9.7)	58.8	(9.2)	61.3	(8.7)	61.7	(8.8)	63.0	(8.0)	*P*<0.001
BMI (kg/m^2)	26.1	(3.5)	26.0	(3.2)	26.2	(3.2)	26.5	(3.0)	27.2	(3.2)	*P*<0.001	25.6	(3.8)	25.8	(4.0)	26.1	(4.3)	26.2	(4.5)	26.9	(4.3)	*P*<0.001
Iron intake (mg/d)†	13.1	(3.6)	13.1	(4.1)	13.1	(3.6)	13.4	(3.7)	13.9	(4.6)	*P*=0.006	11.0	(3.5)	11.2	(3.3)	11.1	(3.4)	10.9	(3.5)	10.5	(3.4)	*P*=0.042
n (%)																						
Menopausal Status																						*P*<0.001
Pre-mp	-	-	-	-	-	-	-	-	-	-		94	(25.6)	49	(13.5)	33	(9.0)	34	(9.4)	19	(5.2)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		26	(7.1)	21	(5.8)	12	(3.3)	9	(2.5)	8	(2.2)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		71	(19.3)	76	(21.0)	53	(14.5)	47	(13.0)	54	(14.8)	
Post-mp	-	-	-	-	-	-	-	-	-	-		176	(48.0)	216	(59.7)	268	(73.2)	271	(75.1)	283	(77.8)	
HRT																						*P*=0.25
Current User	-	-	-	-	-	-	-	-	-	-		70	(19.1)	82	(22.6)	63	(17.2)	53	(14.7)	62	(17.0)	
Former User	-	-	-	-	-	-	-	-	-	-		42	(11.4)	39	(10.8)	52	(14.2)	46	(12.7)	43	(11.8)	
Never Used	-	-	-	-	-	-	-	-	-	-		255	(69.5)	241	(66.6)	251	(68.6)	262	(72.6)	259	(71.2)	
Smoking											*P*=0.77											*P*=0.82
Current smoker	36	(13.0)	26	(9.4)	36	(13.0)	35	(12.6)	32	(11.6)		49	(13.4)	43	(11.9)	42	(11.5)	41	(11.4)	47	(12.9)	
Former smoker	143	(51.6)	154	(55.6)	148	(53.4)	159	(57.4)	148	(53.4)		127	(34.6)	110	(30.4)	111	(30.3)	120	(33.2)	121	(33.2)	
Never smoked	98	(35.4)	97	(35.0)	93	(33.6)	83	(30.0)	97	(35.0)		191	(52.0)	209	(57.7)	213	(58.2)	200	(55.4)	196	(53.9)	
Physical activity											*P*=0.09											*P*=0.27
Inactive	91	(32.8)	85	(30.7)	75	(27.1)	88	(31.7)	87	(31.4)		105	(28.6)	108	(29.8)	116	(31.7)	124	(34.4)	136	(37.4)	
Mod. inactive	47	(17.0)	71	(25.6)	63	(22.7)	65	(23.5)	83	(30.0)		117	(31.9)	110	(30.4)	121	(33.1)	114	(31.6)	121	(33.2)	
Mod. active	67	(24.2)	58	(20.9)	66	(23.8)	59	(21.3)	57	(20.6)		82	(22.3)	87	(24.0)	77	(21.0)	72	(19.9)	65	(17.9)	
Active	72	(26.0)	63	(22.8)	73	(26.4)	65	(23.5)	50	(18.0)		63	(17.2)	57	(15.8)	52	(14.2)	51	(14.1)	42	(11.5)	
Family history of OP	8	(2.9)	11	(4.0)	5	(1.8)	6	(2.2)	8	(2.9)	*P*=0.58	23	(6.3)	20	(5.5)	22	(6.0)	26	(7.2)	22	(6.0)	*P*=0.92
Steroids	14	(5.1)	8	(2.9)	8	(2.9)	6	(2.2)	7	(2.5)	*P*=0.32	14	(3.8)	15	(4.1)	18	(4.9)	13	(3.6)	19	(5.2)	*P*=0.79
Calcium supp.	3	(1.1)	4	(1.4)	6	(2.2)	5	(1.8)	2	(0.7)	*P*=0.64	19	(5.2)	22	(6.1)	22	(6.0)	21	(5.8)	21	(5.8)	*P*=0.99
Vitamin D supp.	61	(22.0)	62	(22.4)	78	(28.2)	56	(20.2)	61	(22.0)	*P*=0.22	119	(32.4)	140	(38.7)	124	(33.9)	103	(28.5)	119	(32.7)	*P*=0.07

Values are means (standard deviations) or numbers (frequencies).

6.4.2 Associations between iron and heel ultrasound

Associations between the bone density parameters BUA (in dB/MHz) and VOS (in m/s) with dietary iron intake and serum ferritin concentrations are presented in **Figure 6.4** for men and in **Figure 6.5** for women. The results are discussed in detail below. Briefly, we found that dietary iron intake was significantly positively associated with BUA in women only.

6.4.2.1 Dietary iron intake and heel ultrasound

In univariate analyses, dietary iron intake correlated significantly and positively with BUA and VOS in women (both r=0.08, *P*<0.05), although no such associations were found in men.

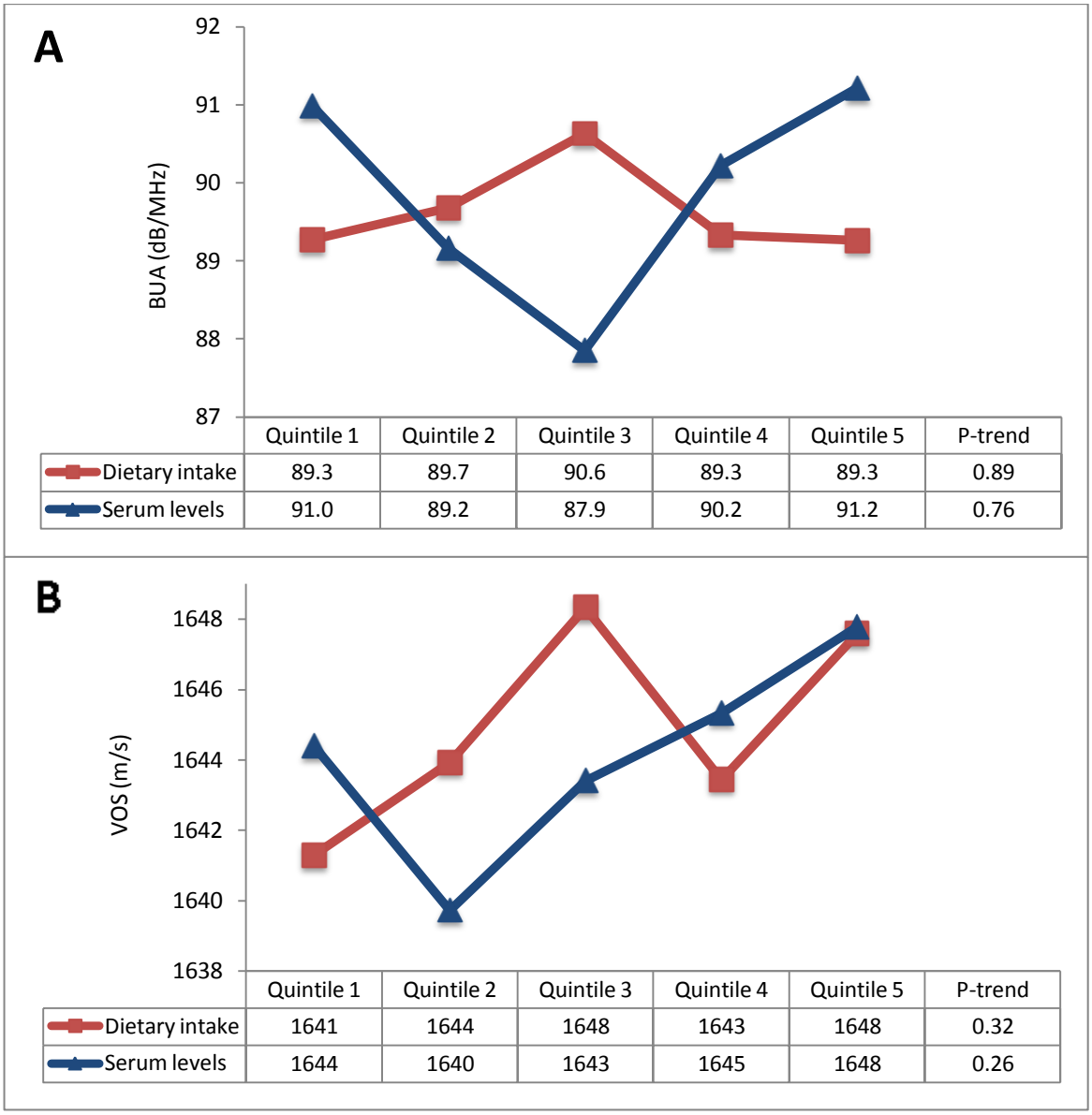
In concordance with the findings from the univariate analyses, multivariate-adjusted linear regression analyses reported that quintiles of dietary iron intake were not associated with measures of heel ultrasound in men. However, a significant linear relationship between increasing quintiles of dietary iron intake and higher BUA was found in women, even after adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids, menopausal status, HRT use, energy intake, dietary calcium intake and the use of calcium and vitamin D supplements (β 0.66 dB/MHz per quintile, *P*-trend=0.045; **Figure 6.5**). Moreover, BUA was 4.4% higher in women in quintile 5 compared to those in the lowest quintile of dietary iron intake (β 3.08 dB/MHz, *P*=0.036), and the 3.6% difference between quintile 4 compared to quintile 1 almost reached statistical significance (β 2.55 dB/MHz, *P*=0.062). Despite the positive correlation between iron intake and VOS in women in univariate analyses, there was no linear trend in multivariate-adjusted regression analyses. Moreover, there was no significant difference in VOS between women of the upper quintiles compared to those of the lowest quintile of dietary iron intake, although the 0.41% difference in VOS between women of quintile 4 compared to quintile 1 was almost significant (β 6.57 m/s, *P*=0.054).

6.4.2.2 Serum ferritin and heel ultrasound

In univariate analyses, serum ferritin concentrations did not correlate with measures of heel ultrasound in men or women.

The results from the multivariate-adjusted regression analyses investigating the relationship between sex-specific quintiles of serum ferritin concentrations with BUA and VOS are also shown in Figures 6.4-6.5. There were no significant associations between serum ferritin levels and heel ultrasound in participants of this cohort. The categorisation of participants into quintiles of serum ferritin differed between men and women, with a finer discrimination in women. For example, men in quintile 1 had a serum ferritin concentration of 9-49 ng/ml and this reflected serum levels of women in quintiles 1 and 2 (8-25 and 25-41 ng/ml, respectively).

Figure 6.4: Associations between dietary iron intake and serum ferritin concentrations with mean BUA **(A)** and VOS **(B)** in men.

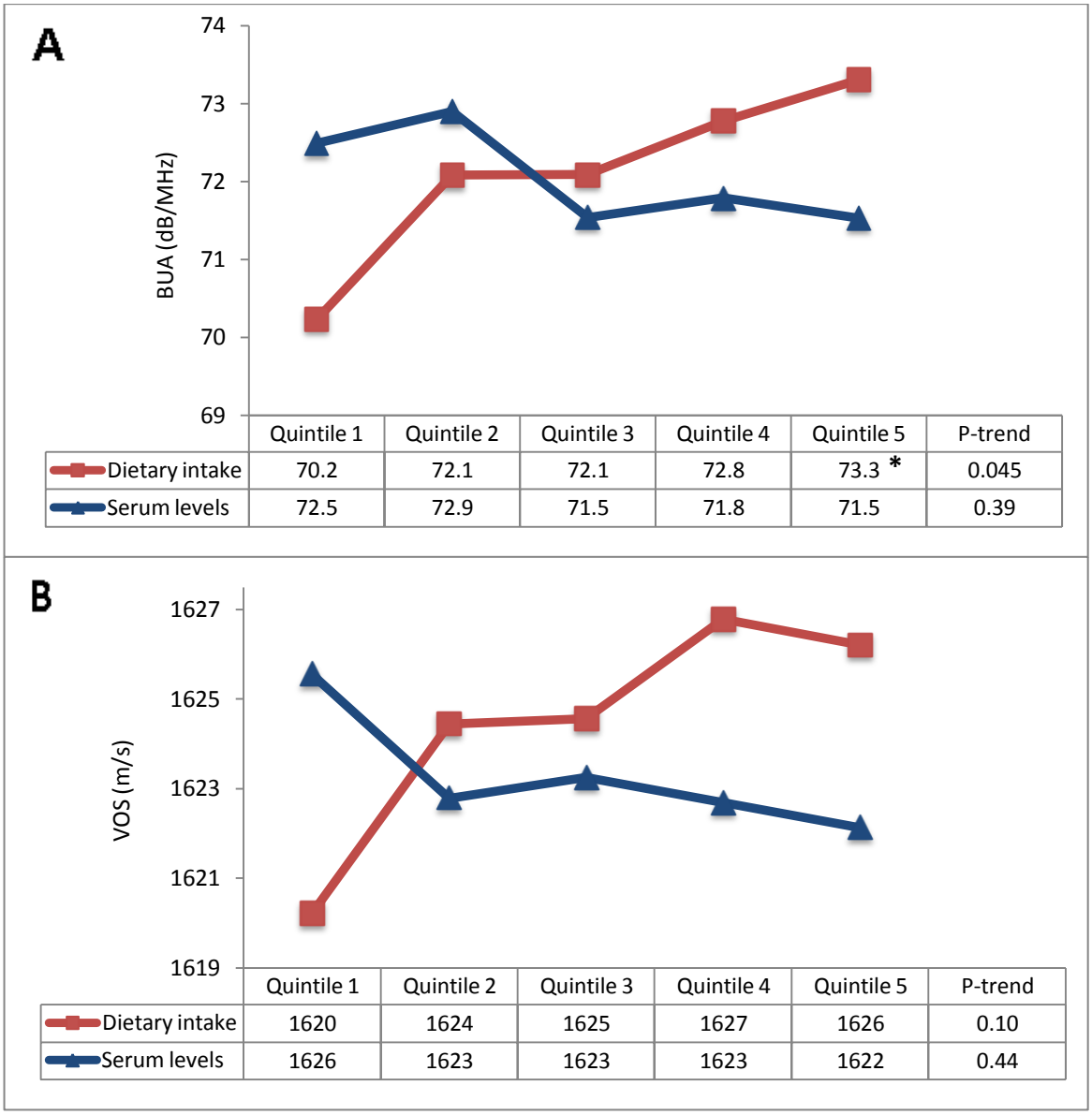


	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	89.3	89.7	90.6	89.3	89.3	0.89
Serum levels	91.0	89.2	87.9	90.2	91.2	0.76

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	1641	1644	1648	1643	1648	0.32
Serum levels	1644	1640	1643	1645	1648	0.26

The mean iron intake for quintile 1 and 5 ranged from 9-20 mg/d. Mean serum ferritin concentrations for quintile 1 and 5 ranged from 32-242 ng/ml. The standard error of the mean (SE) was 1.3-1.5 dB/MHz for BUA and 2.8-3.4 m/s for VOS. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. There were no significant differences between the two upper quintiles referent to quintile 1. n=968 for iron intake and n=682 for serum ferritin.

Figure 6.5: Associations between dietary iron intake and serum ferritin concentrations with mean BUA **(A)** and VOS **(B)** in women.



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	70.2	72.1	72.1	72.8	73.3 *	0.045
Serum levels	72.5	72.9	71.5	71.8	71.5	0.39

	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Dietary intake	1620	1624	1625	1627	1626	0.10
Serum levels	1626	1623	1623	1623	1622	0.44

*The mean iron intake for quintile 1 and 5 ranged from 7-16 mg/d. Mean serum ferritin concentrations for quintile 1 and 5 ranged from 17-142 ng/ml. The standard error of the mean (SE) was 0.9-1.1 dB/MHz for BUA and 2.2-2.7 m/s for VOS. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05. n=1359 for iron intake and n=910 for serum ferritin.*

6.4.3 Associations between iron and fracture risk

In the case-cohort sub-sample of EPIC-Norfolk participants, there were 112 hip fractures, 78 spine fractures and 70 wrist fractures in men, and 339 hip fractures, 124 spine fractures and 218 wrist fractures in women. In the case-cohort that investigated participants with a fracture at any of these three fracture sites (total fracture), there were 248 and 616 fractures in men and women, respectively. The results of the calculation of hazard ratios of fracture risk according to dietary iron intake and serum ferritin concentrations are discussed below. Briefly, both iron intake and serum ferritin were significantly inversely associated with the risk of spine fractures in women only.

6.4.3.1 Iron characteristics of participants with or without a fracture

Women who remained free from fractures over the median 12.6-year follow up had significantly higher mean iron intake from foods compared to those women with a fracture (11±3.4 *vs.* 10.5±3.5 mg/d, *P*=0.003; **Table 6.3**). No such differences were found in men, and there were no significant differences in mean serum ferritin concentrations between fracture and non-fracture subjects in either sex.

Table 6.3: Dietary iron intake and serum ferritin in subjects with and without a total fracture.

	Subjects without a fracture				Subjects with a fracture				
	n	**Mean**	**(SD)**	**[Range]**	***n***	**Mean**	**(SD)**	**[Range]**	***P***
Men									
Dietary iron intake (mg/d)	1709	13.3	(4.0)	[3.4; 42.2]	248	13.5	(4.5)	[5.7; 37.1]	0.39
Serum ferritin levels (ng/ml)	1212	115.4	(83.6)	[8.5; 447.2]	173	117.6	(95.1)	[9.1; 439.4]	0.76
Women									
Dietary iron intake (mg/d)	2138	11.0	(3.4)	[1.9; 29.7]	616	10.5	(3.5)	[3.7; 27.0]	0.003
Serum ferritin levels (ng/ml)	1409	63.6	(52.2)	[8; 442.9]	411	68.5	(53.9)	[8; 296.6]	0.10

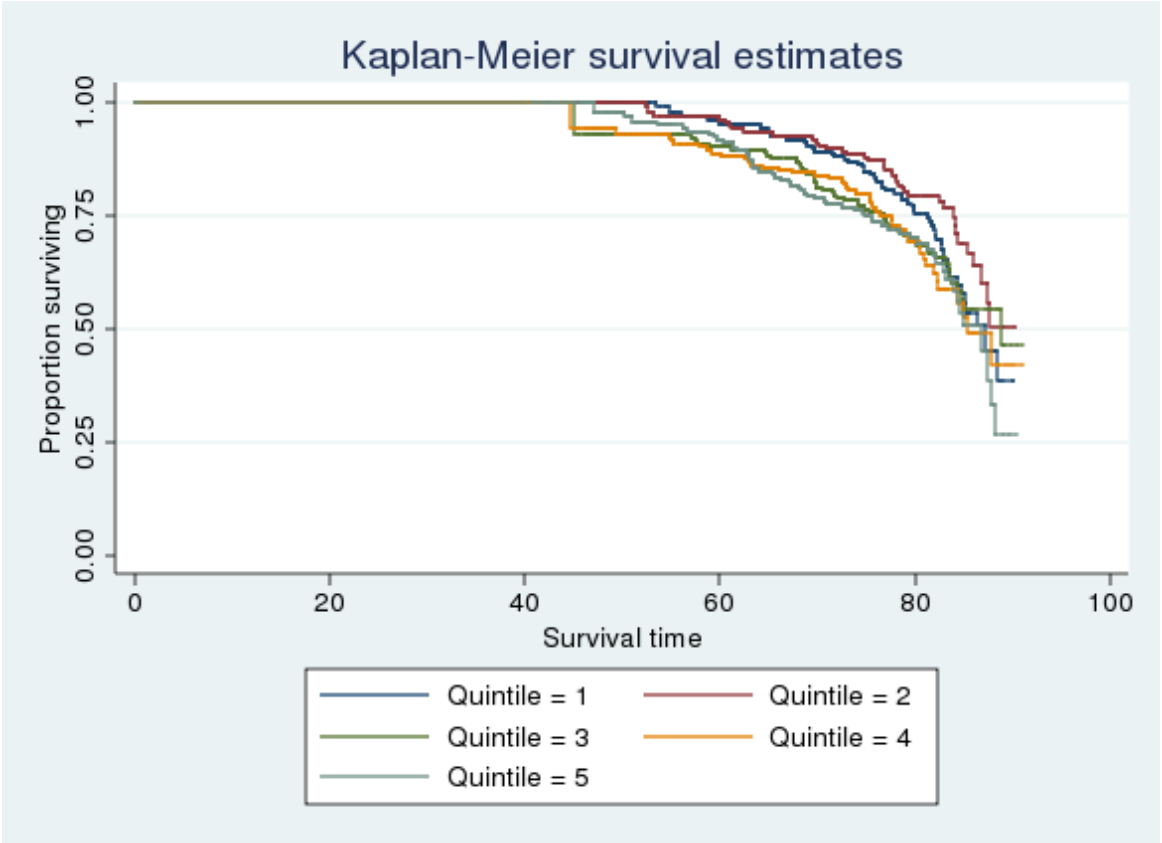
6.4.3.2 Dietary iron intake and fracture risk

In men, the Kaplan Meier plot showed that there was both overlap and cross-over between the five quintiles of dietary iron intake and no one quintile diverged significantly from the others **(Figure 6.6)**. The log-rank test for equality confirmed these observations, showing that total osteoporotic fracture incidence did not differ significantly according to dietary iron intake. In concordance with these findings, the results from the unadjusted as well as fully adjusted Prentice-weighted Cox proportional hazard ratios showed that dietary iron intake was not associated with fracture risk at any site in men **(Table 6.4)**.

In women, the Kaplan Meier plot showed that total fracture incidence in quintile 4 of dietary iron intake appeared to diverge markedly from the other quintiles **(Figure 6.7)**, and the log-rank test for equality confirmed this observation (*P*=0001). In contrast to men, higher dietary iron intake was significantly associated with a reduced fracture risk in women **(Table 6.5)**. Associations were present for spinal fracture risk, where a significant inverse trend was found across the quintile groups (HR 0.85, 95%CI 0.73-0.99, *P*-trend=0.041), after adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroid medication, menopausal status, HRT use, energy intake, dietary calcium intake and the use of calcium and vitamin D supplements. Moreover, women in the upper quintiles of dietary iron intake had significantly lower multivariate-adjusted hazard ratios of spine fracture risk compared to those women in quintile 1 (quintile 4: HR 0.51, 95%CI 0.29-0.92, *P*=0.025; quintile 5: HR 0.41, 95%CI 0.21-0.79, *P*=0.008). Although quintile 4 of dietary iron intake was also significantly associated with a reduction in hip fracture risk, the association lost statistical significance following the adjustment for all covariates. Dietary iron intake was not associated with wrist fractures in this cohort. When investigating hip, spine and wrist fractures combined (total fracture), women in quintile 4 had a significantly lower hazard ratio than those women with the lowest iron intakes (HR 0.65, 95%CI 0.47-0.92, P=0.014), and this remained significant after multivariate adjustment.

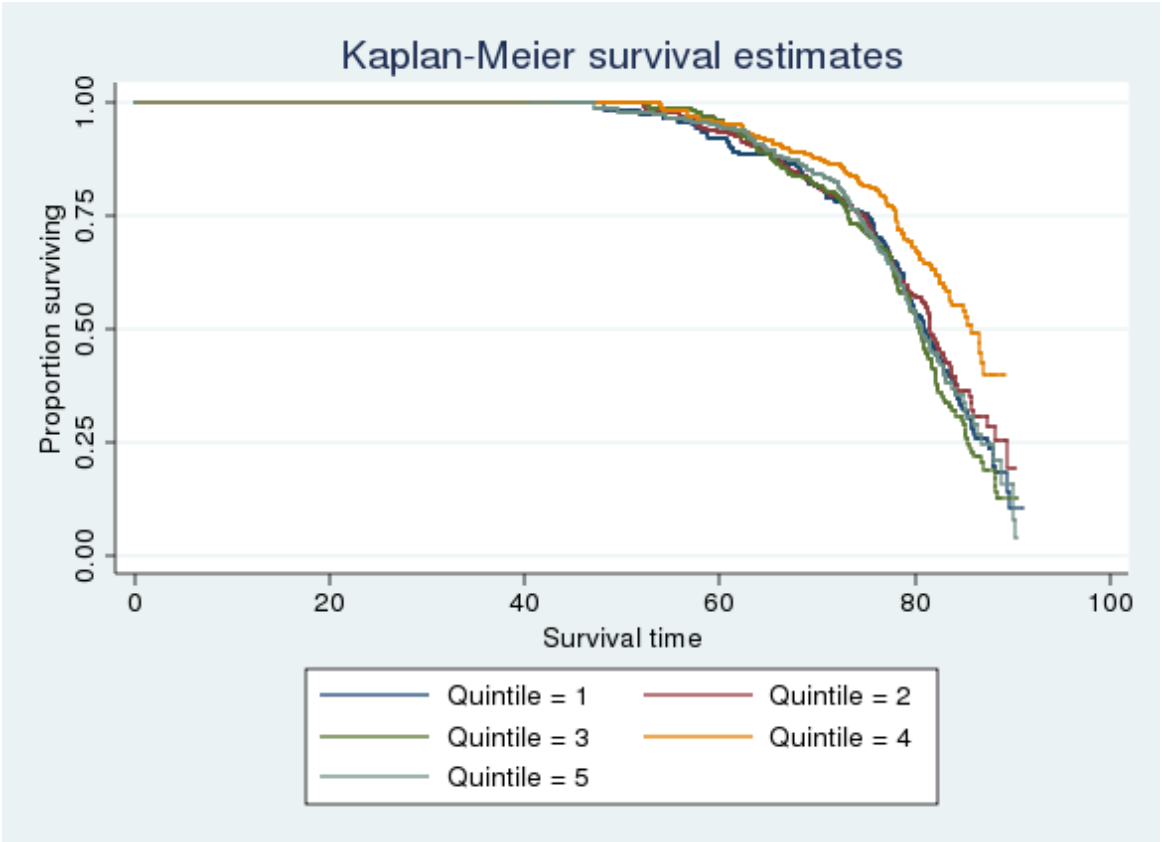
The categorisation of participants into quintiles of dietary iron intake differed between men and women, with a finer discrimination in women. For example, the dietary iron intakes of men in quintile 1 (3.4-10.1 mg/d) were similar to those of women in quintiles 1 and 2 (1.9-8.2 and 8.3-9.6 mg/d, respectively).

Figure 6.6: Kaplan-Meier plot of total fractures by quintiles of dietary iron intake in men.



There were no significant differences between the quintile groups according to the log-rank test for equality (P=0.12). n=1957.

Figure 6.7: Kaplan-Meier plot of total fractures by quintiles of dietary iron intake in women.



The quintile groups differed significantly according to the log-rank test for equality (P=0.001). n=2754.

Table 6.4: Associations between dietary iron intake and fracture risk in men of the EPIC-Norfolk case-cohort.

		Dietary iron intake (mg/d)									
		Quintile 1		**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		3.4 – 10.1		**10.2 – 11.9**		**12.0 – 13.7**		**13.8 – 16.1**		**16.2 - 42.2**	
		n = 392		***n = 391***		***n = 392***		***n = 391***		***n = 391***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[56]		[40]		[50]		[46]		[56]	
	Unadjusted	1.00	0.70	(0.45-1.09)	0.92	(0.60-1.40)	1.02	(0.66-1.56)	1.21	(0.80-1.82)	*P*=0.15
	Model 1	1.00	0.73	(0.46-1.14)	0.92	(0.60-1.41)	0.99	(0.64-1.53)	1.20	(0.80-1.81)	*P*=0.19
	Model 2	1.00	0.72	(0.46-1.15)	0.90	(0.56-1.46)	0.97	(0.59-1.58)	1.12	(0.67-1.86)	*P*=0.36
Hip fracture	[Events]	[30]		[19]		[24]		[21]		[18]	
	Unadjusted	1.00	0.65	(0.35-1.20)	0.84	(0.47-1.50)	1.03	(0.56-1.89)	0.80	(0.43-1.49)	*P*=0.91
	Model 1	1.00	0.71	(0.37-1.33)	0.84	(0.47-1.52)	1.00	(0.54-1.87)	0.81	(0.43-1.53)	*P*=0.86
	Model 2	1.00	0.80	(0.41-1.56)	1.00	(0.51-1.98)	1.17	(0.57-2.40)	0.92	(0.45-1.90)	*P*=0.80
Spinal fracture	[Events]	[15]		[11]		[18]		[13]		[21]	
	Unadjusted	1.00	0.75	(0.34-1.67)	1.28	(0.63-2.59)	1.11	(0.52-2.40)	1.75	(0.89-3.46)	*P*=0.07
	Model 1	1.00	0.77	(0.35-1.74)	1.28	(0.62-2.62)	1.06	(0.49-2.27)	1.72	(0.87-3.40)	*P*=0.09
	Model 2	1.00	0.79	(0.33-1.88)	1.31	(0.58-2.94)	1.11	(0.44-2.83)	1.84	(0.77-4.39)	*P*=0.12
Wrist fracture	[Events]	[13]		[9]		[13]		[13]		[22]	
	Unadjusted	1.00	0.66	(0.28-1.54)	0.96	(0.44-2.11)	0.95	(0.44-2.08)	1.70	(0.85-3.38)	*P*=0.08
	Model 1	1.00	0.64	(0.27-1.51)	0.95	(0.43-2.11)	0.90	(0.41-1.94)	1.70	(0.82-3.17)	*P*=0.09
	Model 2	1.00	0.53	(0.23-1.26)	0.73	(0.31-1.73)	0.65	(0.29-1.45)	0.98	(0.42-2.29)	*P*=0.75

Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. No significant differences between the two upper quintiles referent to the lowest quintile. Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity and use of steroids. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 1957 for total fracture, n 1842 for hip fracture, n 1808 for spine fracture, n 1806 for wrist fracture.

Table 6.5: Associations between dietary iron intake and fracture risk in women of the EPIC-Norfolk case-cohort.

		Dietary iron intake (mg/d)									
		Quintile 1		**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		1.9 – 8.2		**8.3 – 9.6**		**9.7 – 11.0**		**11.1 – 13.1**		**13.2 – 29.7**	
		n = 551		***n = 551***		***n = 551***		***n = 551***		***n = 550***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[151]		[123]		[137]		[89]		[116]	
	Unadjusted	1.00	0.92	(0.69-1.24)	1.14	(0.85-1.52)	**0.61**	**(0.45-0.83)****	1.01	(0.74-1.36)	*P*=0.24
	Model 1	1.00	0.91	(0.67-1.23)	1.11	(0.83-1.50)	**0.60**	**(0.44-0.82)****	1.01	(0.74-1.38)	*P*=0.25
	Model 2	1.00	0.95	(0.69-1.30)	1.20	(0.87-1.66)	**0.65**	**(0.47-0.92)***	1.12	(0.78-1.62)	*P*=0.61
Hip fracture	[Events]	[79]		[73]		[77]		[43]		[67]	
	Unadjusted	1.00	1.17	(0.80-1.71)	1.37	(0.94-1.99)	**0.62**	**(0.41-0.94)***	1.29	(0.87-1.90)	*P*=0.89
	Model 1	1.00	1.13	(0.76-1.67)	1.28	(0.87-1.89)	**0.60**	**(0.39-0.91)***	1.32	(0.89-1.96)	*P*=0.91
	Model 2	1.00	1.21	(0.81-1.81)	1.44	(0.95-2.19)	0.68	(0.44-1.07)	1.57	(0.98-2.52)	*P*=0.65
Spinal fracture	[Events]	[44]		[14]		[27]		[22]		[17]	
	Unadjusted	1.00	0.35	(0.19-0.66)	0.73	(0.44-1.23)	**0.56**	**(0.33-0.95)***	**0.48**	**(0.27-0.86)***	***P*=0.044**
	Model 1	1.00	0.35	(0.19-0.67)	0.76	(0.45-1.29)	0.59	(0.33-1.03)	**0.50**	**(0.27-0.91)***	*P*=0.08
	Model 2	1.00	0.33	(0.18-0.62)	0.67	(0.37-1.20)	**0.51**	**(0.29-0.92)***	**0.41**	**(0.21-0.79)****	***P*=0.041**
Wrist fracture	[Events]	[51]		[45]		[49]		[34]		[39]	
	Unadjusted	1.00	1.00	(0.65-1.52)	1.14	(0.75-1.73)	0.72	(0.46-1.13)	0.93	(0.60-1.45)	*P*=0.38
	Model 1	1.00	0.97	(0.63-1.50)	1.09	(0.71-1.66)	0.69	(0.43-1.09)	0.87	(0.55-1.37)	*P*=0.24
	Model 2	1.00	0.99	(0.63-1.57)	1.16	(0.73-1.83)	0.74	(0.45-1.22)	0.95	(0.56-1.62)	*P*=0.48

*Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. Significant differences between the two upper quintiles referent to the lowest quintile: * (P<0.05), ** (P<0.01). Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids, menopausal status and HRT. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 2754 for total fracture, n 2525 for hip fracture, n 2334 for spine fracture, n 2409 for wrist fracture.*

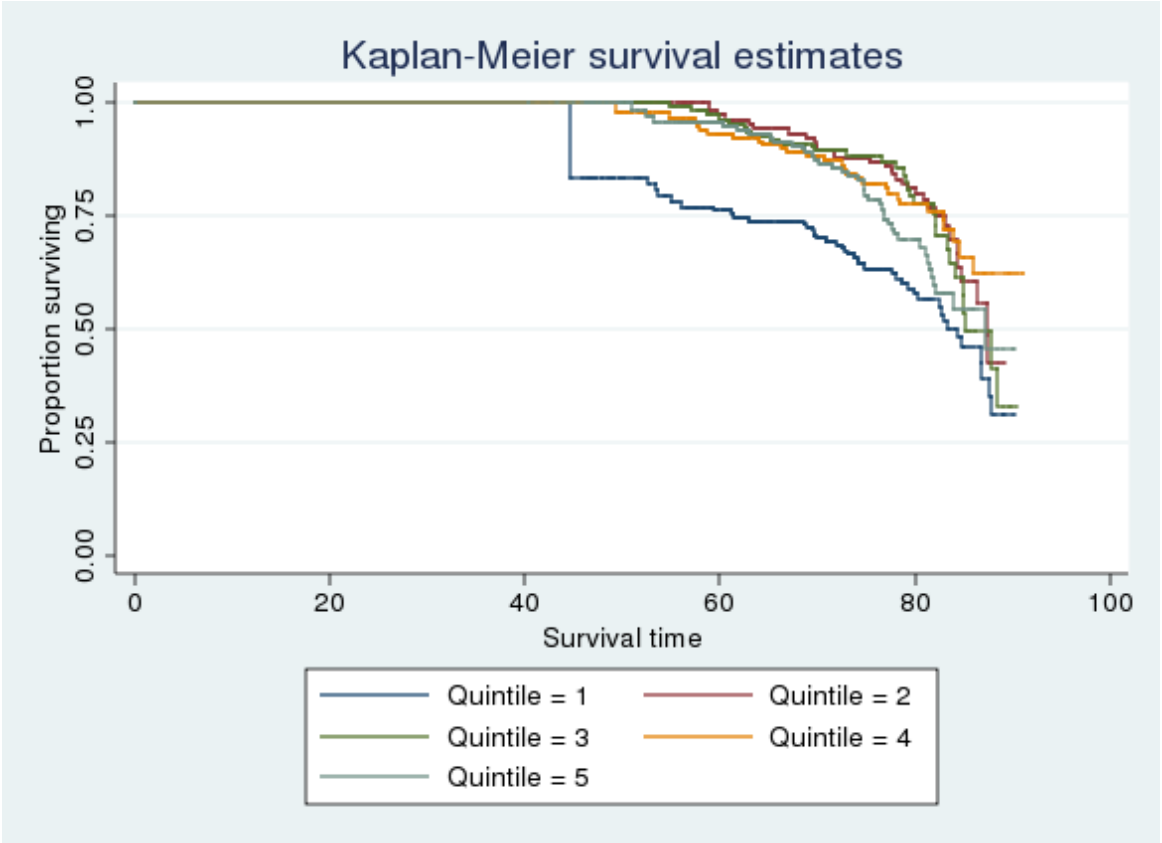
6.4.3.3 Serum ferritin concentrations and fracture risk

In men, the Kaplan Meier plot demonstrated a great risk of total fracture incidence in quintile 1 of serum ferritin compared to the higher quintiles **(Figure 6.8)**. However, the log-rank test for equality showed that this was not statistically significant (*P*=0.22). These findings demonstrate that men in serum ferritin quintile 1 had a high number of fractures early during the follow-up period compared to those men in the other quintiles, and at the end of the follow-up (March 2009), the number of fractures were comparable between all quintile groups of serum ferritin. The results from the Prentice-weighted Cox proportional hazard ratio analysis also showed that quintiles of serum ferritin concentrations were not associated with fracture risk at any site in men **(Table 6.6)**.

In women, the Kaplan Meier plot showed that the number of total fractures in quintile 1 of serum ferritin markedly diverged from that of the other quintiles, which was statistically significant using the log-rank test for equality (*P*=0.018, **Figure 6.9**). Moreover, in women serum ferritin concentrations were significantly inversely associated with spinal fracture risk (HR 0.78, 95%CI 0.65-0.94, *P*-trend=0.009), even after the adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroid medication, menopausal status, HRT use, energy intake, dietary calcium intake and the use of calcium and vitamin D supplements **(Table 6.7)**. Moreover, the multivariate-adjusted risk of fractures at the spine was also significantly lower in women of quintile 4 (HR 0.30, 95%CI 0.14-0.64, *P*=0.002) and quintile 5 (HR 0.44, 95%CI 0.22-0.87, *P*=0.018) compared to those women in the lowest quintile of serum ferritin. Serum ferritin levels were not associated with hip, wrist and total fracture in women.

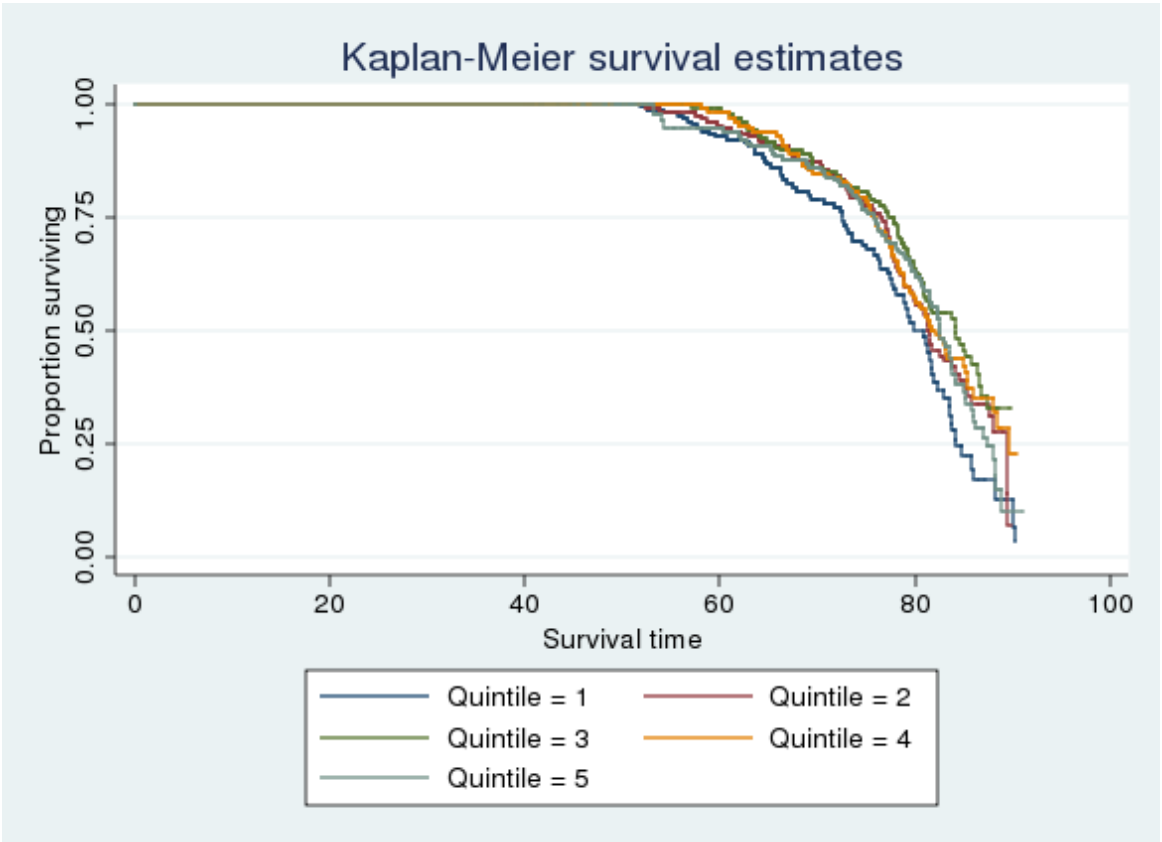
As with dietary intakes of iron, the categorisation of participants into quintiles of serum ferritin also differed between men and women, with a finer discrimination in women. For example, ferritin in quintile 1 in men (8.5-47.8 ng/ml) was comparable to that of quintiles 1 and 2 in women (8.0-25.1 and 25.2-41.7 ng/ml, respectively).

Figure 6.8: Kaplan-Meier plot of total fractures by quintiles of serum ferritin in men.



There were no significant differences between the quintile groups according to the log-rank test for equality (P=0.22). n=1385.

Figure 6.9: Kaplan-Meier plot of total fractures by quintiles of serum ferritin in women.



The quintile groups differed significantly according to the log-rank test for equality (P=0.018). n=1820.

Table 6.6: Associations between serum ferritin and fracture risk in men of the EPIC-Norfolk case-cohort.

		Serum ferritin levels (ng/ml)								
		Quintile 1		**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		8.5 – 47.8		**47.9 – 76.0**		**76.1 – 113.2**		**113.3 – 169.2**		**169.3 - 447.2**	
		n = 277		***n = 277***		***n = 277***		***n = 277***		***n = 277***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[42]		[32]		[32]		[28]		[39]	
	Unadjusted	1.00	0.72	(0.43-1.20)	0.81	(0.49-1.34)	0.63	(0.37-1.07)	1.00	(0.62-1.63)	*P*=0.82
	Model 1	1.00	0.69	(0.41-1.16)	0.76	(0.45-1.26)	0.62	(0.36-1.04)	0.94	(0.57-1.53)	*P*=0.67
	Model 2	1.00	0.68	(0.40-1.15)	0.78	(0.46-1.31)	0.58	(0.33-1.00)	0.88	(0.53-1.47)	*P*=0.51
Hip fracture	[Events]	[18]		[17]		[14]		[14]		[16]	
	Unadjusted	1.00	0.93	(0.45-1.89)	0.91	(0.44-1.91)	0.77	(0.36-1.64)	1.05	(0.51-2.15)	*P*=0.89
	Model 1	1.00	0.89	(0.42-1.88)	0.88	(0.41-1.92)	0.79	(0.36-1.72)	1.03	(0.49-2.18)	*P*=0.92
	Model 2	1.00	0.88	(0.40-1.94)	1.09	(0.48-2.44)	0.69	(0.30-1.57)	0.91	(0.40-2.08)	*P*=0.64
Spinal fracture	[Events]	[13]		[9]		[6]		[8]		[14]	
	Unadjusted	1.00	0.70	(0.29-1.65)	0.51	(0.19-1.34)	0.63	(0.25-1.54)	1.19	(0.55-2.56)	*P*=0.79
	Model 1	1.00	0.68	(0.29-1.63)	0.48	(0.18-1.28)	0.61	(0.25-1.48)	1.08	(0.49-2.39)	*P*=0.93
	Model 2	1.00	0.68	(0.29-1.64)	0.47	(0.16-1.31)	0.63	(0.25-1.58)	1.13	(0.50-2.56)	*P*=0.87
Wrist fracture	[Events]	[14]		[7]		[11]		[9]		[9]	
	Unadjusted	1.00	0.49	(0.19-1.26)	0.77	(0.34-1.75)	0.61	(0.26-1.43)	0.64	(0.27-1.53)	*P*=0.43
	Model 1	1.00	0.46	(0.18-1.20)	0.69	(0.31-1.56)	0.56	(0.24-1.32)	0.57	(0.24-1.33)	*P*=0.29
	Model 2	1.00	0.47	(0.18-1.25)	0.72	(0.32-1.61)	0.57	(0.23-1.40)	0.56	(0.22-1.39)	*P*=0.31

Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. No significant differences between the two upper quintiles referent to the lowest quintile. Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity and use of steroids. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 1385 for total fracture, n 1304 for hip fracture, n 1277 for spine fracture, n 1283 for wrist fracture.

Table 6.7: Associations between serum ferritin and fracture risk in women of the EPIC-Norfolk case-cohort.

		Serum ferritin levels (ng/ml)								
		Quintile 1	**Quintile 2**		**Quintile 3**		**Quintile 4**		**Quintile 5**	
		8.0 – 25.1	**25.2 – 41.7**		**41.8 – 61.3**		**61.4 – 94.4**		**94.5 – 442.9**	
		n = 367	***n = 362***		***n = 366***		***n = 361***		***n = 364***	
		HR (ref)	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	[Events]	[76]		[75]		[76]		[89]		[95]	
	Unadjusted	1.00	0.70	(0.47-1.04)	0.58	(0.39-0.86)	0.68	(0.46-1.00)	0.69	(0.47-1.02)	*P*=0.15
	Model 1	1.00	0.72	(0.48-1.07)	0.61	(0.41-0.92)	0.69	(0.47-1.03)	0.73	(0.50-1.08)	*P*=0.22
	Model 2	1.00	0.72	(0.48-1.08)	0.62	(0.42-0.93)	0.71	(0.48-1.05)	0.73	(0.49-1.08)	*P*=0.23
Hip fracture	[Events]	[36]		[35]		[41]		[59]		[60]	
	Unadjusted	1.00	0.69	(0.40-1.18)	0.62	(0.37-1.05)	0.89	(0.55-1.47)	0.87	(0.54-1.42)	*P*=0.76
	Model 1	1.00	0.67	(0.39-1.17)	0.65	(0.38-1.11)	0.89	(0.53-1.49)	0.92	(0.56-1.52)	*P*=0.60
	Model 2	1.00	0.68	(0.39-1.19)	0.66	(0.38-1.14)	0.93	(0.55-1.55)	0.93	(0.57-1.54)	*P*=0.57
Spinal fracture	[Events]	[22]		[17]		[13]		[12]		[18]	
	Unadjusted	1.00	0.58	(0.30-1.13)	0.36	(0.17-0.74)	**0.30**	**(0.15-0.63)****	**0.45**	**(0.23-0.86)***	***P*=0.012**
	Model 1	1.00	0.61	(0.31-1.19)	0.38	(0.18-0.81)	**0.31**	**(0.15-0.65)****	**0.44**	**(0.22-0.86)***	***P*=0.009**
	Model 2	1.00	0.62	(0.32-1.20)	0.38	(0.18-0.81)	**0.30**	**(0.14-0.64)****	**0.44**	**(0.22-0.87)***	***P*=0.009**
Wrist fracture	[Events]	[29]		[27]		[26]		[29]		[33]	
	Unadjusted	1.00	0.74	(0.42-1.29)	0.60	(0.34-1.04)	0.65	(0.37-1.13)	0.69	(0.40-1.19)	*P*=0.23
	Model 1	1.00	0.74	(0.42-1.30)	0.61	(0.34-1.07)	0.67	(0.38-1.17)	0.75	(0.43-1.31)	*P*=0.37
	Model 2	1.00	0.74	(0.42-1.31)	0.62	(0.35-1.09)	0.68	(0.39-1.20)	0.74	(0.42-1.30)	*P*=0.37

*Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. Significant differences between the two upper quintiles referent to the lowest quintile: * (P<0.05), ** (P<0.01). Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity and use of steroids. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 1820 for total fracture, n 1673 for hip fracture, n 1538 for spine fracture, n 1585 for wrist fracture.*

6.5 Discussion

To the best of my knowledge, these data are the first to investigate both the potential prospective associations between dietary iron intakes and iron status with the long-term fracture risk; and the cross-sectional associations between iron intake and bone health in a large population of British men and women. Following multivariate adjustment, the results from the cross-sectional study showed that dietary iron intake, but not serum ferritin concentrations, were significantly and positively associated with BUA in women; and the prospective investigations showed that both higher dietary iron intake and higher serum ferritin concentrations were significantly inversely associated with fracture risk in women, particularly spine fractures. In contrast to women, in men, there were no associations between iron intake or serum status with heel ultrasound and fracture risk.

6.5.1 Heel ultrasound

In the cross-sectional study, there were no associations between serum ferritin and heel ultrasound, but associations with dietary iron intake were sex-specific, with significant associations found in women. For iron intake, there was a marginal linear relationship with BUA in women; and mean intakes of 16 mg/d compared to 7 mg/d were significantly associated with 4.4% higher BUA. The difference in mean iron intake between the extreme quintiles (9 mg/d) can be achieved through the usual diet, although particular attention should be paid to consuming a variety of iron-rich foods. For example, three whole ready-to-eat apricots (equivalent to one portion) and ten cashews as snack foods, combined with four tablespoons of green or brown lentils and four spears of broccoli as part of a main meal have an iron content of approximately 9 mg [145, 350]. The cross-sectional findings of a positive association between dietary iron intake and measures of heel ultrasound potentially reflect the important role of iron in bone health. It is well documented that iron plays a crucial role as a cofactor in the hydroxylation reactions within collagen fibres [8, 10], which increases overall collagen strength [136], as well as in the synthesis of vitamin D [172, 383], an important mediator in calcium absorption [125]. Our findings are in agreement with two US studies of a small number of women (n<250) which reported similar findings with BMD [142, 181]. The effect sizes of 4-14% higher BMD between extreme quartiles of iron intake were dependent on the BMD site [181], but were comparable to the present findings of 4.4% with BUA. A direct comparison of the absolute values for the effect sizes was not possible, as no observational study has previously used measures of heel ultrasound. Despite the agreement with previous studies, the present investigations were conducted in a much larger sample of women (n=1359 *vs.* n<250), and thus may provide more robust findings compared to those of previous studies. Moreover, to the best of my knowledge, this study is the first cross-sectional investigation of a potential association between dietary iron

intake and bone health in men, as previous studies were only undertaken in small female populations [142, 181, 406].

In contrast to dietary intakes of iron, serum ferritin concentrations as an indicator of iron stores in the body were not associated with measures of heel ultrasound in both men and women in this cohort. To date, only two comparable cross-sectional studies have been published and those reported contradictory findings [182, 409]. In comparison to the present iron status investigations in 1592 British participants aged 60±10 years, the studies were undertaken in 2943 older South Korean men and women (mean age: 72±11 years) and 5148 Korean men and women with a large age range of 10-95 years (mean age: 45±19 years), respectively. In the first investigation, the results were sex-specific, with significant positive associations between serum ferritin levels and multiple BMD sites found only in men [182]. In this study, mean serum ferritin concentrations were higher in both men and women than those of the present study population (men: 128±230 *vs.* 114±79 ng/ml; women: 77±123 *vs.* 63±51 ng/ml) which may, at least in part, explain the contradictory findings. In the second study, the results were also sex-specific, but were significant in women [409]. Moreover, that study found potentially detrimental effects of higher iron status for bone health in older women, with the highest compared to the lowest serum ferritin concentrations being significantly associated with 3.4% lower BMD and an increased risk for osteoporosis (OR 1.55, 1.09-2.23) and fractures (OR 1.52, 1.02-2.27). However, these inverse associations were only in women older than 45 years, and the authors suggested that the significant drop in oestrogen levels associated with women of this age group may partly explain these findings. The contradictory cross-sectional results of the present investigations compared to the two previous studies in Korean populations highlight the importance for more epidemiological studies which investigate potential associations between markers of iron status and bone health in large populations of men and women.

Interestingly, in our study, ferritin levels increased significantly with higher dietary iron intakes in men, but levels decreased significantly in women. To the best of my knowledge, this is a novel finding, and it may be a result of the differences in age-related ferritin levels which are independent of diet. In our study, those women with the highest iron intake in quintile 5, but who had much lower serum ferritin levels compared to women with the lowest intake in quintile 1, were significantly younger and less likely to be post-menopausal. There is a vast body of evidence to show that ferritin concentrations are much lower during the reproductive ages in women, but levels tend to increase two to three fold following menopause, possibly as a result of ceased menses [375, 381, 410]. In contrast to women, there is no difference in ferritin across age groups in men, with levels being relatively steady at all ages [382], and hence the significant decrease in age across quintiles of iron intake in this study was most likely not a determining factor of serum ferritin in men. The present findings may also be an explanation for the small association between iron intake and ferritin concentrations, which has been shown to be

especially low in women. For example, in this study, their correlation coefficient was significant in men (r=0.16; *P*<0.05) but not in women (r=-0.02; *P*>0.05); and this is in agreement with previous studies which found either very weak (r=0.14-0.15, *P*≤0.03) or no correlation between dietary iron and ferritin [223, 377-382]. Further explanations for these weak relationships include variations in the biological availability of iron from foods, iron losses and the use of iron supplements [379]. Moreover, in the present study, the small association between the two iron measures may provide an explanation for the differing associations that were observed between heel ultrasound and dietary intakes of iron and serum ferritin.

Another potential reason for the different associations of iron intake and ferritin with heel ultrasound in the present study may relate to the differing numbers of pre- and postmenopausal women in the quintiles in our study, although menopausal status was adjusted for in the multivariate model. In the iron intake investigations, where significant positive associations with heel ultrasound were found, the number of pre- and postmenopausal women was relatively equally distributed between the quintiles. However, in the serum ferritin investigations, where there were no significant associations, quintile 1 had the highest number of pre-menopausal women (13%) and the lowest number of post-menopausal women (54%), but numbers were vice versa in quintile 5 (0% premenopausal women, 88% postmenopausal women). Postmenopausal status compared to pre-menopausal has previously been associated with significantly lower BMD due to a high rate of bone loss during the menopausal transition [80, 411]. Thus, the high percentage of postmenopausal women in the top quintile of serum ferritin in this study may partly explain the absence of a relationship between serum ferritin and heel ultrasound in women. This is in spite of the positive relationship between indices of body fat distribution and ferritin levels, as reported in numerous epidemiological studies [412-414]. Obesity is associated with changes in iron metabolism leading to alterations in iron status, including low serum iron concentrations (hypoferraemia), although the aetiology is uncertain. In agreement with this, those men and women in our study with the highest serum ferritin levels had significantly higher BMI than participants with the lowest levels, and higher BMI has previously been shown to be protective for bone health [84, 112]. However, in the present study, the detrimental effects of increasing age and being post-menopausal, as previously discussed, are likely to have outweighed the protective effects of higher BMI and serum ferritin levels on bone health, and this may partially explain the lack of association between ferritin and heel ultrasound.

In conclusion, the present cross-sectional investigations found significant associations between higher dietary intakes of iron and higher BUA in women but not in men, but serum ferritin concentrations as an indicator of iron status were not associated with measurements of heel ultrasound in this population. More epidemiological studies in both men and women and

with large sample sizes are needed to investigate whether the present findings are indeed sex-specific.

6.5.2 Fracture risk

To date, only a few prospective studies have been published and those available have focused on investigating associations between dietary iron intake and bone loss [186, 405], or associations between iron status and bone loss and short-term fracture risk [185]. However, to the best of my knowledge, no previous studies have examined the potential role of iron in reducing long-term fracture risk. Thus, the present prospective investigations of iron intake and status with long-term fracture risk in a large sample of older British men and women are completely novel. We found that both iron intake and serum ferritin concentrations in association with fracture risk were sex-specific, with significant associations only found in women. For dietary iron intakes, we reported that women with intakes of 12.0±0.6 mg/d compared to those with the lowest intakes of 6.9±1.0 mg/d had a 35% lower total fracture risk (fracture risk of the hip, spine and wrist combined) after the median 12.6 years follow-up. There was also a significant linear trend between higher iron intake and lower spine fracture risk in these women. Moreover, spine fracture risk was 49% and 59% lower in women with intakes of 12.0±0.6 mg/d and 16.0±2.8 mg/d compared to the lowest intakes of 6.9±1.0 mg/d, respectively. When comparing women with all total fractures and those who remained free from fractures during follow-up, we found that women, who developed fractures, reported significantly lower mean dietary iron intakes at baseline (10.5±3.5 *vs.* 11.0±3.4 mg/d). Higher serum ferritin concentrations were also significantly associated with lower fracture risk in women but not in men in this cohort. The association between higher ferritin and a reduction in spine fracture risk was found to be linear; and women with mean serum ferritin concentrations of 73.9±9.1 ng/ml and 144.2±57.3 ng/ml compared to the lowest concentrations of 16.3±4.9 ng/ml had a significantly reduced spine fracture risk of 70% and 56%, respectively. In contrast to women, there were no significant associations between dietary intakes and status of iron with fracture risk in men in this cohort.

To date, only one prospective study has investigated iron status and fracture risk [185], and their findings of a potential detrimental association between higher serum ferritin concentrations and increased fracture risk in women are in disagreement with our results, which suggest a beneficial effect in the same sex. This study in 1729 Korean middle-aged men and women had a much shorter follow-up of only three years compared to the median follow-up of 12.6 years in the present study. Moreover, the populations' mean ferritin concentration was higher in both men and women compared to that of our study population (men: 147±84 *vs.* 114±79 ng/ml; women: 77±51 *vs.* 63±51 ng/ml), and this may explain some of the discrepancies in results.

The reason for the present sex-specific findings of significant associations between iron intake and serum ferritin with fracture risk in women may partly be due to the smaller number of fracture events in men, which is consistent with the published literature [22]. In this case-cohort sub-sample, there were 6%, 4% and 4% of fractures at the hip, spine and wrist respectively in men compared to 13%, 5% and 9% of fractures respectively in women. The present investigations may thus have had more power to detect potential associations between intake and status measures of iron and fracture risk in women than in men. Moreover, our sex-specific findings may also be explained by differences in dietary iron intakes between men and women. Although the mean serum ferritin concentrations in men (116±85 ng/ml) and women (65±53 ng/ml) were within the normal ranges of 20-300 and 15-150 ng/ml, respectively [376], there were sex-specific differences in adequate intakes of dietary iron. Most men (91%) met the RNI of 8.7 mg/d, whereas only 51% of older women and 13% of menstruating women up to the age of 50 years met their respective RNIs of 8.7 and 14.8 mg/d. Moreover, less than 1% of men did not meet the LRNI of 4.7 mg/d, whereas a fairly large number of younger women (14%) did not meet the LRNI of 8 mg/d. Thus, women in this cohort had a much wider range of dietary iron intakes than men, and this may partly explain our sex-specific results of significant associations in women only. The differences in iron intakes may also relate to the different categorisation of men and women into quintiles of iron. For example, the dietary iron intakes of men in quintile 1 (3.4-10.1 mg/d) reflected those of women in quintiles 1 and 2 (1.9-8.2 and 8.3-9.6 mg/d, respectively). The same pattern was also found for serum ferritin concentrations, where ferritin in quintile 1 in men (8.5-47.8 ng/ml) was comparable to that of quintiles 1 and 2 in women (8.0-25.1 and 25.2-41.7 ng/ml, respectively). The finer discrimination of quintiles of both iron intake and serum ferritin in women may be another explanation for why significant inverse associations between iron and fracture risk were only found in women.

In conclusion, the present investigation shows that lower dietary iron intakes and serum ferritin concentrations as an indicator of iron status were a significant predictor of a higher fracture risk in women, and future prospective studies of large mixed populations are needed to confirm the present sex-specific novel findings.

6.5.3 Strengths and limitations

The cross-sectional study of heel ultrasound provides a number of potential advantages over previous epidemiological studies; whereas our prospective investigations of fracture risk are completely novel. The inclusion of both men and women in the study population has provided novel data of a cross-sectional association between iron intake and bone health. To the best of my knowledge, only two previous cross-sectional studies included both men and women in their investigations of iron status and BMD [182, 409]. Moreover, our work of iron intake and heel ultrasound addressed limitations of small sample sizes, which had previously ranged from 159-

244 participants. In contrast, our study comprised 2327 men and women, and thus was better powered to detect potential diet-bone associations. To date, there is data from only two cross-sectional studies of iron status and bone health in Asian populations, which are comparable to our investigations, and those had contradictory findings [182, 409]. Our study of serum ferritin concentrations and heel ultrasound provides the first data from a Western population, although we did not find a relationship between iron status and bone health in either sex. Our prospective investigations of dietary iron intake and serum ferritin concentrations as an indicator of iron status with long-term fracture risk are novel. To date, there is data from only one study which has investigated the role of iron status on short-term fracture risk [185]. The results of a potential role of both higher dietary iron intakes and higher serum ferritin concentrations in fracture risk reduction in a large sample of women are thus novel findings.

Although our work had a robust study design, it also had a number of limitations. The cross-sectional study design of the bone density analyses only examined relations between diet and bone density for a single point in time. The positive associations reported in women suggest that there was a relation between dietary intakes of iron and heel ultrasound, but conclusions about the influence of iron on bone health cannot be drawn. Similarly, the prospective study design of the fracture analyses was limited by the inability to identify possible secular changes in dietary iron intakes and iron status over the follow-up period and subsequent exposure misclassification, as data were only available from the 7-day food diaries and blood samples taken at baseline. Moreover, the fracture data had been obtained from hospital admissions which are most likely underestimated for spine fractures due to a large absence in their clinical attention and radiologic detection [168, 293, 294]. This may have reduced the power of the present study to detect the associations between iron intake or serum ferritin concentrations and spine fracture risk. Although multivariate adjustment models were applied in the analyses, a number of other relevant confounders previously associated with bone health, including sunlight exposure [295], were not measured as part of the EPIC-Norfolk study. Furthermore, residual confounding may have occurred despite the adjustment for covariates and may have resulted in bias in exposure effect estimates.

6.6 Conclusion

The present cross-sectional investigations found that higher dietary intakes of iron were significantly associated with 4.4% higher BUA in women, but not in men, in this cohort. These differences in bone density between women with low and high iron intakes may have important implications for the development of fractures in the long term. In fact, in our prospective study we found that higher dietary intakes of iron were significantly associated with up to 49% lower spine fracture risk as well as 35% lower total fracture risk (hip, spine and wrist fracture risk combined) in these women. Moreover, higher serum ferritin concentrations as an indicator of iron status were a significant predictor of up to 70% reduced spine fracture risk in women. The present findings highlight the importance of an adequate iron intake from foods and iron status in women. With iron deficiency anaemia being the most prevalent nutrient deficiency worldwide and women having a particularly high risk of developing osteoporosis and associated fractures with increasing age, women should ensure an adequate intake of iron from foods as this may be an important strategy in long-term fracture prevention. The present study provides novel prospective data on the long-term fracture risk with iron intake and status, and addresses a number of limitations of previous cross-sectional studies on bone density including a large sample size and the use of a British study population of men and women. Future studies should conduct RCTs to investigate the effects of iron intake on bone density and long-term fracture risk, as this has not been conducted before. These studies will be crucial for confirming the present sex-specific findings of a potential beneficial role of iron in preventing osteoporosis and fractures in women.

CHAPTER 7

THE ROLE OF IRON INTAKE FROM DIFFERENT FOOD SOURCES IN BONE HEALTH

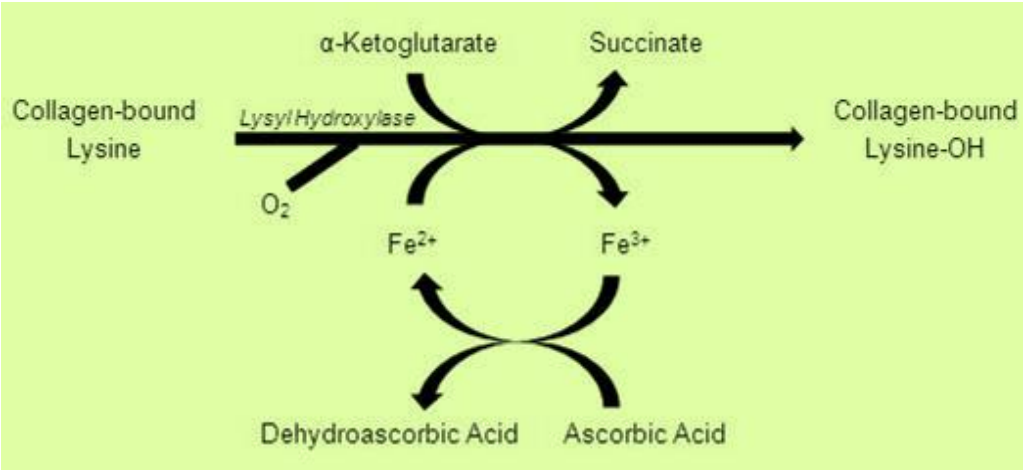
7.1 Abstract

Iron is crucial for bone collagen synthesis and vitamin D synthesis. In the previous chapter, epidemiological associations between iron intake and body iron stores with indicators of bone health were investigated and the results showed that iron was a significant predictor of higher heel ultrasound and reduced spine fracture risk in women, but not in men. However, the dietary intake of iron provides no information on its bioavailability, which differs between the different chemical forms of iron, thereby potentially affecting the underlying mechanisms differently. It may be suggested that animal compared to plant iron intake may be stronger associated with measures of bone health due to its higher absorption rate. To date, the role of iron in bone health has only been studied independent of the food source, and hence the present investigations are completely novel. We aimed to explore i) potential cross-sectional associations between dietary iron intake from different sources and measures of heel ultrasound and ii) potential prospective associations with fracture risk in a sub-set of the 25,639 EPIC-Norfolk men and women aged 39-79 years at baseline. The results from the cross-sectional study showed that iron intake from plant sources was significantly associated with up to 5.8% higher BUA and 0.5% higher VOS in women only. The largest difference in mean plant iron intake between the highest and lowest group in women was 8 mg/d, and this is achievable through the usual diet, although particular attention should be paid to consuming a variety of iron-rich foods. In the prospective study, the highest *vs.* the lowest iron intake from animal sources was significantly associated with 56% lower spine fracture risk in women, but with increased hip fracture risk in men (HR 2.29, 95%CI 1.11-4.73). These data are completely novel as previous studies have only investigated iron intake independent of the food source. Our findings suggest that the different food sources of iron intake may need to be taken into consideration in future epidemiological studies of iron and bone health, as these provide important information on the bioavailability of iron.

7.2 Introduction

In the previous chapter (Chapter 6, page 143), associations between dietary iron intake and bone health, amongst others, were investigated and the results showed that higher iron intakes were significantly associated with higher heel ultrasound measurements and a reduction in spine fracture risk in women only. These sex-specific findings of a potential beneficial role of iron in bone health may reflect its two cofactor roles which are relevant to bone: the synthesis of bone collagen and the synthesis of vitamin D. Firstly, iron is an essential activator of enzymes involved in the hydroxylation of prolyl and lysyl residues within collagen fibres [8, 10] **(Figure 7.1)**. In this reaction, ferrous iron is oxidised to ferric iron, activating lysyl and prolyl hydroxylase to form collagen hydroxylysine and hydroxyproline in the process, respectively. The subsequent formation of covalent bonds between adjacent collagen fibres leads to stronger collagen cross-links, thus increasing overall collagen strength [136]. As both bone and cartilage contain a structurally stable network of collagen, impaired collagen synthesis resulting from inadequate iron intake may potentially be a risk factor for the development of osteoporosis and associated fractures.

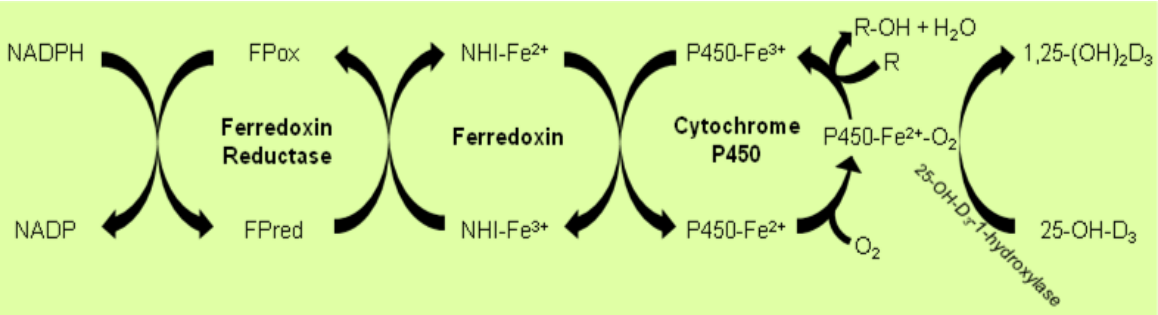
Figure 7.1: The hydroxylation of lysine in collagen fibres.



Adapted from Medeiros & Wildman (2011) [324].

Secondly, iron is crucial to the conversion of 25-hydroxyvitamin D into its active form 1,25-dihydroxycholecalciferol in the kidneys **(Figure 7.2)**. Iron in the form of ferredoxin acts as a cofactor to the reaction-specific enzyme 25-hydroxycholecalciferol-1-hydroxylase [172, 383]. Vitamin D is an important mediator of calcium homeostasis by increasing calcium absorption efficiency, and calcium is one of the fundamental bone-forming compounds [125]. An insufficient dietary iron intake may thus also play a role in osteoporosis and fracture development through compromised mineralisation of bone tissue.

Figure 7.2: The role of iron in vitamin D synthesis.



Adapted from DeLuca (1976) [172] and Jones et al. (1998) [383].

Despite the significant associations between higher dietary iron intake and higher heel ultrasound and a reduction in fracture risk in women in this study, the dietary intake of iron provides no information on its bioavailability, which differs between the different chemical forms of iron [188-192, 358, 359, 415], thereby potentially affecting the underlying mechanisms differently. Iron exists in two forms: haem and non-haem. Haem iron is a derivative of haemoglobin and myoglobin of meat and fish, whereas non-haem iron is present as iron salts in foods. Animal sources of iron including red meat, poultry and fish contain both chemical forms, with less than 40% present as haem-iron [187]. In contrast, plant-based sources of iron including whole grains and fruit and vegetables only contain the non-haem form of iron. To date, the role of iron in bone health has only been studied independent of the food source [142, 181, 186, 405-407]. Therefore, it is unclear whether or not iron from plant- and animal-based sources may vary in their underlying mechanisms. It may be suggested that, if plant and animal iron were to act differently, this may be a result of their differing levels of intestinal absorption. Animal-based haem iron is much better absorbed than iron in its non-haem form (15-40% *vs.* 1-15%) [188-192]. Moreover, the level of absorption of non-haem iron is highly dependent on factors such as the iron content of the meal, an individual's iron status and the presence of absorption inhibitors including phytates and polyphenols, and absorption enhancers including the reducing agent vitamin C [358, 359, 415]. In contrast, the absorption of haem iron is not affected by any of these factors. Furthermore, potential differences in haem and non-haem iron intake are most likely not reflected beyond the intestinal absorption of iron. This is because there is no differentiation between the different iron sources in the metabolism and transport of iron. During absorption, haem iron enters the intestinal cell as an intact compound separated from its haemoprotein (i.e. haemoglobin or myoglobin). Once in the enterocyte, haem is cleaved by haem oxygenase, thereby releasing iron which enters a pool of intracellular iron from non-haem sources [357]. Iron, bound to the plasma protein Tf, is then released into the circulation [370]. Therefore, haem iron is the greater contributor towards the body's iron pool resulting from its higher level of absorption despite non-haem iron making out a greater percentage of total dietary iron intake [193]. The consequences of this for bone health are not known. However, it may be suggested that animal-

based haem iron may potentially be contributing more towards the underlying mechanisms of bone health than non-haem iron due to the differences in intestinal absorption. To date, no studies have investigated potential differences in iron sources in their role in bone health.

7.2.1 Chapter aims and objectives

In order to address some of these limitations, this chapter aimed to:

i) Investigate potential cross-sectional associations between dietary iron intake from different sources and the heel ultrasound parameters BUA and VOS.

ii) Examine potential prospective associations between dietary iron intake from different sources and the risk of fracture at the hip, spine and wrist in a British population of men and women aged between 39 and 79 years at baseline.

This study will provide novel investigations of potential differences between animal- and plant-based iron and their role in bone health. It was hypothesised that animal compared to plant iron intake may be stronger associated with higher heel ultrasound and a reduced risk of fractures due to its higher absorption rate.

7.3 Methods

As described in detail in Chapter 2 (page 40), two types of studies were undertaken on a randomly-selected sample of men and women of the EPIC-Norfolk study. Briefly, the cross-sectional study of heel ultrasound was based on a random sub-cohort of 4000 participants who had attended the first health check, and the prospective investigations of fracture risk were based on a case-cohort design using the same subset of 4000 participants and a set of 1502 participants who had experienced a fracture up to 31st March 2009.

Initially, the EPIC-Norfolk dataset included data on total dietary iron intake, but the relative contributions of plant and animal sources of iron were unknown. Thus, I created a new group of variables, in collaboration with one other colleague, which identified the following food sources: plant, animal (land), animal (marine) and animal-derived. We each coded all of the 11,326 food items according to their food sources. Foods with multiple food sources received multiple responses. We then checked our coding against each other and agreed on the final coding for each food item. We were responsible for the decisions we made. Following the initial coding, members of staff at EPIC-Norfolk combined the group of variables into sensible food source combinations. The new dataset included four categories of food sources which were specific to iron intake as well as 17 categories of unclassified foods and mixed dishes. All categories were based on iron intake in mg/d. I then calculated iron intake for the different sources according to these categories. For the purpose of this chapter, the calculation of iron

intake from plant and animal sources included only those food categories with obvious plant or animal sources. Categories were not included in the calculation if they contained foods with an unknown contribution of iron from plant and animal sources. I calculated the intake of plant iron from the plant category which exclusively contained plant-based foods including fruits, vegetables, grains and concentrated squashes. Animal iron intake was estimated from six food categories: animal land (e.g. beef and chicken), animal marine (e.g. fish and seafood), animal-derived (e.g. milk and eggs) and three categories of unclassified foods which contained a large number of mainly animal-based mixed dishes including meat and offal dishes, savoury pies, and sandwich fillings and soups with meat.

All analyses were stratified by sex. Subjects were excluded from the subsequent analyses if they had missing data for the 7dDD and covariate information, and if they had suffered a fracture at a different site and were not part of the random sub-cohort. Firstly, iron intake was calculated separately for total dietary iron intake and for the plant and animal-based sources using means and standard deviations, as all variables were relatively normally distributed. Then, the percentage contribution of animal iron towards total dietary iron intake was calculated, and this is hereafter referred to as the % animal ratio. Differences in mean estimates of iron from different sources between men and women were determined using paired *t*-tests. To assess differences in the correlation between iron intake from different food sources with total dietary iron intake and serum ferritin concentrations, Pearson correlation coefficients were determined. The latter were also used to determine the correlation between measurements of heel ultrasound and iron. Next, participants were grouped into two sets of quintiles according to their mean iron intake from plant sources, animal sources or the % animal ratio. For the cross-sectional study of heel ultrasound, associations between quintiles of iron sources and broadband ultrasound attenuation (BUA) and velocity of sound (VOS) as measures of heel ultrasound were determined using multiple regressions. BUA is an indicator of the structural organisation of bone, whereas VOS determines bone stiffness [(63)]. Then, potential prospective associations between quintiles of iron sources and fracture risk at the hip, spine, wrist and their combined total were investigated using Prentice-weighted Cox proportional hazard ratios [(221)]. For both the cross-sectional and the prospective studies, potential associations between the top and the lowest quintile were investigated. The investigations from chapter 7 of associations between total dietary iron intake and i) heel ultrasound and ii) fracture risk were also repeated in this chapter for comparison reasons. All analyses adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements.

7.4 Results

Following the exclusion of 608 subjects (11.4%) from the subsequent analyses as previously discussed, the study was performed in 4711 participants, 58% of which were women. Participants were on average (mean±SD) 60±10 years old and had a mean BMI of 26.3±3.9 kg/m^2. In the 1385 men and 1819 women with blood measurements, mean serum ferritin was significantly higher in men compared to women (115.7±85.1 *vs.* 64.7±52.6 ng/ml, *P*<0.001).

7.4.1 Cohort descriptives

7.4.1.1 The relationship between iron by food source and total dietary iron

Plant foods contributed more towards total dietary iron intake than animal-based foods **(Table 7.1)**, although the relative contributions were comparable between men and women. For example, approximately 65% and 67% of iron intake in men and women respectively were associated with plant-based foods, whereas the remaining contributions (35% and 33%) were from animal sources. Despite these similarities, men compared to women had significantly higher plant iron intake (8.7±3.7 *vs.* 7.3±3.1 mg/d), animal iron intake (2.8±1.5 *vs.* 2.1±1.2 mg/d), and total dietary iron intake (13.3±4.1 *vs.* 10.9±3.4 mg/d, *P*<0.001). The ratio of the percentage contribution of animal iron towards total dietary iron intake was also significantly higher in men than in women (21.7±10.2 *vs.* 19.7±9.9, *P*<0.001).

Table 7.1: Dietary iron intake from animal and plant sources.

	Men (*n*=1957)		Women (*n*=2754)		
	Mean	SD	Mean	SD	*P*-value
Total dietary iron intake (mg/d)	13.3	4.1	10.9	3.4	*P*<0.001
Plant iron intake (mg/d)	8.7	3.7	7.3	3.1	*P*<0.001
Animal iron intake (mg/d)	2.8	1.5	2.1	1.2	*P*<0.001
Ratio (% animal)	21.7	10.2	19.7	9.9	*P*<0.001

Ratio is iron intake from animal sources as a percentage of total dietary iron intake.

Pearson correlation coefficients showed that plant-based iron was highly correlated with total dietary iron intake in both men (r=0.89) and women (r=0.90, all *P*<0.05) **(Table 7.2)**. Correlations between animal and total iron intake were also significant, but were only moderate (r=0.33, *P*<0.05 in both sexes). In contrast, serum ferritin showed better correlations with iron intake from animal than plant sources. For example, the correlation between serum ferritin and animal iron was low but significant in both men (r=0.14) and women (r=0.09, all *P*<0.05); whereas plant iron showed no correlation with serum ferritin in men (r=0.02, *P*>0.05) and a small but negative correlation in women (r=-0.06, *P*<0.05). The correlation between animal and plant iron intake was slightly negative and only significant in men (men: r=-0.06, *P*<0.05; women: r=-0.02, *P*>0.05).

Table 7.2: Correlations between iron intake by food source with serum ferritin.

	Men (*n*=1957)				Women (*n*=2754)			
	Plant iron	Animal iron	Ratio (% animal)	Total dietary iron	Plant iron	Animal iron	Ratio (% animal)	Total dietary iron
Animal iron	-0.06*	-	-	-	-0.02	-	-	-
Ratio (% animal)	-0.51*	0.81*	-	-	-0.49*	0.78*	-	-
Total dietary iron	0.89*	0.33*	-0.20*	-	0.90*	0.33*	-0.21*	-
Serum ferritin	0.02	0.14*	0.10*	0.07*	-0.06*	0.09*	0.12*	-0.05*

*Pearson correlation coefficients were significant at *P<0.05.*

7.4.1.2 Descriptive statistics by quintiles of iron intake by food source

Characteristics of the 4711 men and women stratified by quintiles of iron intake by food source are shown in **Table 7.3** for plant iron, **Table 7.4** for animal iron and **Table 7.5** for the animal-to-total-iron ratio. The mean±SD iron intakes from plant sources for the quintile groups were as follows: in men: Q1 4.7±0.9 mg/d, Q2 6.7±0.4 mg/d, Q3 8.1±0.4 mg/d, Q4 9.8±0.6 mg/d and Q5 14.2±3.9 mg/d; and in women: Q1 3.9±0.8 mg/d, Q2 5.5±0.3 mg/d, Q3 6.7±0.3 mg/d, Q4 8.2±0.5 mg/d and Q5 12.0±2.7 mg/d. Higher intakes of plant-based iron was associated with a healthier lifestyle. For example, subjects in quintile 5 were less likely to smoke, more active and had a healthier BMI compared to those in quintile 1 ($P \leq 0.001$). Participants in the top compared to the bottom quintile of plant iron were also younger and more likely to report the use of calcium or vitamin D supplements ($P \leq 0.011$). However, there were no differences in family history of osteoporosis and steroid medication use across quintiles of plant iron ($P \geq 0.13$). In women, serum ferritin levels decreased significantly with each quintile of plant iron intake (β±SE -2.5±0.9 ng/ml, $P=0.005$), but ferritin did not differ between plant iron quintiles in men ($P=0.79$).

For iron intake from animal sources, the mean±SD for the quintile groups were as follows: in men: Q1 1.2±0.4 mg/d, Q2 2.0±0.2 mg/d, Q3 2.5±0.2 mg/d, Q4 3.2±0.3 mg/d and Q5 5.1±1.6 mg/d; and in women: Q1 0.8±0.3 mg/d, Q2 1.4±0.1 mg/d, Q3 1.9±0.1 mg/d, Q4 2.4±0.2 mg/d and Q5 3.8±1.4 mg/d. In contrast to plant iron, higher intake of animal iron was associated with an unhealthier lifestyle. For example, subjects in quintile 5 were more likely to smoke compared to those in quintile 1 ($P \leq 0.042$), and BMI increased significantly across quintiles of animal iron intake (Q5 *vs.* Q1: 27.1 *vs.* 26.0 kg/m^2 in men; 26.7 *vs.* 25.7 kg/m^2; all $P<0.001$). There were no differences in family history of osteoporosis, physical activity, steroid medication use, and calcium or vitamin D supplements across quintiles of animal iron in both sexes ($P \geq 0.16$). Serum ferritin concentrations increased significantly with each increasing quintile of animal iron in both sexes (β±SE 7.2±1.6 ng/ml in men and 4.3±0.9 ng/ml in women; all $P<0.001$).

The mean±SD iron intake by quintile groups for the animal iron ratio were as follows: in men: Q1 9.2±3.2 mg/d, Q2 15.8±1.2 mg/d, Q3 20.4±1.5 mg/d, Q4 26.1±1.8 mg/d and Q5 36.9±7.6 mg/d; and in women: Q1 7.8±2.9 mg/d, Q2 13.9±1.4 mg/d, Q3 18.5±1.3 mg/d, Q4 23.7±1.8 mg/d and Q5 34.6±7.8 mg/d. In agreement with iron from animal sources, the ratio of animal iron to total dietary iron intake was also associated with an unhealthier lifestyle. For example, men and women in quintile 5 were more likely to smoke and had a higher BMI compared to those in quintile 1 ($P<0.001$), and women were also less active and less likely to report the use of vitamin D supplements ($P \leq 0.008$). There were no differences in family history of osteoporosis, the use of steroid medication and calcium supplements between quintiles of the ratio of animal iron to total dietary iron intake in both men and women ($P \geq 0.08$). Serum ferritin concentrations increased significantly with each increasing quintile of the animal iron ratio in both sexes (β±SE 5.1±1.6 ng/ml in men and 5.8±0.9 ng/ml in women; all $P \leq 0.002$).

Table 7.3: Baseline characteristics of the 1957 men and 2754 women stratified by quintiles of plant iron intake.

	Men											Women										
Plant iron intake (mg/d)	**Quintile 1 1.2 – 5.8**		**Quintile 2 5.9 – 7.3**		**Quintile 3 7.4 – 8.8**		**Quintile 4 8.9 – 10.9**		**Quintile 5 11.0 – 39.1**			**Quintile 1 0.0 – 4.9**		**Quintile 2 5.0 – 6.1**		**Quintile 3 6.2 – 7.3**		**Quintile 4 7.4 – 9.1**		**Quintile 5 9.2 – 24.9**		
	n = 392		***n = 391***		***n = 392***		***n = 391***		***n = 391***		***P*-trend**	***n = 551***		***n = 551***		***n = 551***		***n = 551***		***n = 550***		***P*-trend**
Mean (SD)																						
Age (years)	61.2	(9.8)	61.1	(9.5)	59.2	(9.1)	58.7	(9.4)	58.5	(9.6)	*P*<0.001	61.8	(9.8)	60.0	(9.4)	59.7	(9.3)	59.1	(9.4)	58.2	(9.5)	*P*<0.001
BMI (kg/m^2)	26.9	(3.7)	26.6	(3.2)	26.6	(3.1)	26.4	(3.2)	26.1	(3.3)	*P*=0.001	26.8	(4.5)	26.3	(4.5)	26.3	(4.2)	25.8	(4.1)	25.6	(4.2)	*P*<0.001
Animal iron (mg/d)	2.8	(1.6)	2.9	(1.5)	2.9	(1.5)	2.8	(1.5)	2.6	(1.6)	*P*=0.032	2.1	(1.5)	2.1	(1.1)	2.1	(1.0)	2.0	(1.2)	2.0	(1.2)	*P*=0.25
Ratio (% animal)	29.4	(11.4)	24.3	(8.8)	22.0	(8.2)	18.8	(7.5)	13.8	(7.1)	*P*<0.001	27.4	(11.7)	22.0	(8.6)	19.4	(7.6)	16.6	(7.5)	12.9	(6.7)	*P*<0.001
Total iron (mg/d)	9.3	(2.2)	11.3	(1.9)	12.8	(1.9)	14.6	(1.8)	18.6	(4.2)	*P*<0.001	7.4	(1.9)	9.2	(1.6)	10.3	(1.3)	11.8	(1.6)	15.6	(3.0)	*P*<0.001
Serum ferritin (ng/ml)	117.8	(93.6)	108.9	(76.0)	120.5	(86.1)	114.0	(83.4)	117.3	(85.7)	*P*=0.79	68.8	(54.7)	66.5	(50.8)	67.9	(61.8)	61.7	(49.7)	58.9	(44.2)	*P*=0.005
Energy (kcal/d)	1864	(432)	2137	(428)	2257	(446)	2413	(438)	2528	(538)	*P*<0.001	1404	(336)	1618	(335)	1703	(328)	1795	(367)	1898	(365)	*P*<0.001
Fat (g/d)	76.2	(23.7)	84.6	(24.2)	87.6	(25.2)	91.0	(24.9)	91.4	(29.5)	*P*<0.001	57.3	(18.9)	64.1	(20.0)	64.8	(19.4)	67.4	(21.6)	67.7	(20.1)	*P*<0.001
MUFA (g/d)	26.8	(8.6)	29.4	(8.4)	30.7	(9.2)	31.7	(8.9)	31.8	(10.6)	*P*<0.001	19.8	(6.8)	22.1	(6.8)	22.5	(6.8)	23.1	(7.6)	23.6	(7.5)	*P*<0.001
PUFA (g/d)	13.5	(5.1)	15.4	(5.6)	16.7	(6.3)	17.5	(6.1)	18.2	(7.2)	*P*<0.001	10.1	(3.7)	12.0	(4.3)	12.3	(4.2)	13.2	(4.8)	13.5	(4.6)	*P*<0.001
Saturated FA (g/d)	29.4	(10.9)	32.7	(11.3)	32.9	(11.2)	34.3	(11.4)	33.7	(12.4)	*P*<0.001	22.5	(8.8)	24.7	(9.4)	24.5	(8.9)	25.4	(9.8)	25.0	(9.0)	*P*<0.001
n (%)																						
Menopausal Status																						*P*=0.002
Pre-mp	-	-	-	-	-	-	-	-	-	-		67	(12.2)	80	(14.5)	76	(13.8)	91	(16.5)	100	(18.2)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		15	(2.7)	20	(3.6)	24	(4.4)	32	(5.8)	36	(6.6)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		82	(14.9)	85	(15.5)	103	(18.7)	81	(14.7)	97	(17.6)	
Post-mp	-	-	-	-	-	-	-	-	-	-		387	(70.2)	366	(66.4)	348	(63.1)	347	(63.0)	317	(57.6)	
HRT																						*P*=0.15
Current User	-	-	-	-	-	-	-	-	-	-		72	(13.1)	92	(16.7)	92	(16.7)	109	(19.8)	107	(19.5)	
Former User	-	-	-	-	-	-	-	-	-	-		69	(12.5)	61	(11.1)	69	(12.5)	64	(11.6)	61	(11.1)	
Never Used	-	-	-	-	-	-	-	-	-	-		410	(74.4)	398	(72.2)	390	(70.8)	378	(68.6)	382	(69.4)	
Smoking											*P*<0.001											*P*<0.001
Current smoker	80	(20.4)	64	(16.4)	31	(7.9)	33	(8.4)	30	(7.7)		121	(22.0)	86	(15.6)	58	(10.5)	45	(8.2)	33	(6.0)	
Former smoker	214	(54.6)	210	(53.7)	224	(57.1)	226	(57.8)	207	(52.9)		174	(31.6)	157	(28.5)	182	(33.0)	174	(31.6)	202	(36.7)	
Never smoked	98	(25.0)	117	(29.9)	137	(35.0)	132	(33.8)	154	(39.4)		256	(46.4)	308	(55.9)	311	(56.4)	332	(60.2)	315	(57.3)	
Physical activity											*P*<0.001											*P*<0.001
Inactive	158	(40.3)	141	(36.1)	115	(29.3)	105	(26.8)	95	(24.3)		243	(44.1)	183	(33.2)	172	(31.2)	153	(27.8)	156	(28.4)	
Mod. inactive	93	(23.7)	85	(21.7)	107	(27.3)	100	(25.6)	86	(22.0)		149	(27.0)	190	(34.5)	176	(31.9)	189	(34.3)	173	(31.4)	
Mod. active	71	(18.1)	79	(20.2)	90	(23.0)	93	(23.8)	103	(26.3)		97	(17.6)	100	(18.1)	119	(21.6)	126	(22.9)	135	(24.6)	
Active	70	(17.9)	86	(22.0)	80	(20.4)	93	(23.8)	107	(27.4)		62	(11.3)	78	(14.2)	84	(15.3)	83	(15.0)	86	(15.6)	
Family history of OP	8	(2.0)	10	(2.6)	13	(3.3)	14	(3.6)	13	(3.3)	*P*=0.69	32	(5.8)	29	(5.3)	25	(4.5)	36	(6.5)	32	(5.8)	*P*=0.68
Steroids	14	(3.6)	19	(4.9)	16	(4.1)	6	(1.5)	13	(3.3)	*P*=0.13	24	(4.4)	31	(5.6)	19	(3.5)	19	(3.5)	21	(3.8)	*P*=0.33
Calcium supp.	4	(1.0)	2	(0.5)	5	(1.3)	8	(2.1)	6	(1.5)	*P*=0.40	17	(3.1)	34	(6.2)	24	(4.4)	42	(7.6)	38	(6.9)	*P*=0.006
Vitamin D supp.	64	(16.3)	79	(20.2)	93	(23.7)	102	(26.1)	92	(23.5)	*P*=0.011	128	(23.2)	165	(30.0)	182	(33.0)	199	(36.1)	201	(36.6)	*P*<0.001

Abbreviations: Mp, menopausal; Family history of OP, family history of osteoporosis; Supp., supplements.

Values are means (standard deviations) or numbers (frequencies).

Table 7.4: Baseline characteristics of the 1957 men and 2754 women stratified by quintiles of animal iron intake.

Animal iron intake (mg/d)	Men											Women										
	Quintile 1 0.1 – 1.6 *n = 392*		Quintile 2 1.7 – 2.2 *n = 391*		Quintile 3 2.3 – 2.8 *n = 392*		Quintile 4 2.9 – 3.7 *n = 391*		Quintile 5 3.8 – 16.4 *n = 391*		***P*-trend**	Quintile 1 0.8 – 1.1 *n = 551*		Quintile 2 1.2 – 1.6 *n = 551*		Quintile 3 1.7 – 2.1 *n = 551*		Quintile 4 2.2 – 2.7 *n = 551*		Quintile 5 2.8 – 24.3 *n = 550*		***P*-trend**
Mean (SD)																						
Age (years)	60.1	(10.1)	60.0	(9.4)	60.5	(9.4)	59.6	(9.5)	58.5	(9.2)	*P*=0.018	58.8	(10.1)	60.3	(9.6)	59.8	(9.3)	60.2	(9.3)	59.7	(9.3)	*P*=0.17
BMI (kg/m^2)	26.0	(3.2)	26.1	(3.0)	26.5	(3.2)	26.7	(3.3)	27.1	(3.6)	*P*<0.001	25.7	(4.2)	25.8	(4.0)	26.2	(4.5)	26.3	(4.4)	26.7	(4.4)	*P*<0.001
Plant iron (mg/d)	9.3	(4.4)	8.6	(3.5)	8.7	(3.6)	8.3	(3.2)	8.6	(3.6)	*P*=0.004	7.3	(3.1)	7.4	(3.3)	7.2	(2.8)	7.3	(3.3)	7.3	(3.0)	*P*=0.68
Ratio (% animal)	10.9	(5.1)	17.1	(4.8)	20.9	(5.6)	25.7	(6.5)	33.8	(9.5)	*P*<0.001	9.2	(4.7)	15.3	(4.9)	19.2	(5.3)	23.2	(6.6)	31.4	(9.7)	*P*<0.001
Total iron (mg/d)	12.3	(4.5)	12.4	(3.6)	13.0	(3.8)	13.4	(3.4)	15.6	(4.1)	*P*<0.001	9.7	(3.3)	10.3	(3.2)	10.6	(2.9)	11.2	(3.4)	12.5	(3.5)	*P*<0.001
Serum ferritin (ng/ml)	99.9	(77.0)	114.2	(88.9)	114.6	(79.2)	113.4	(77.3)	136.6	(98.3)	*P*<0.001	55.4	(46.5)	59.5	(43.3)	66.8	(53.5)	69.5	(58.2)	72.0	(57.9)	*P*<0.001
Energy (kcal/d)	2034	(486)	2152	(471)	2203	(466)	2352	(497)	2457	(530)	*P*<0.001	1544	(396)	1618	(348)	1668	(343)	1760	(381)	1828	(388)	*P*<0.001
Fat (g/d)	74.1	(22.5)	81.8	(24.3)	84.1	(22.7)	92.7	(27.0)	98.2	(27.0)	*P*<0.001	56.3	(20.5)	60.3	(18.3)	63.8	(17.7)	68.8	(19.9)	72.1	(21.1)	*P*<0.001
MUFA (g/d)	25.4	(8.1)	28.3	(8.4)	29.4	(8.3)	32.6	(9.3)	34.7	(9.6)	*P*<0.001	19.1	(7.3)	20.7	(6.5)	22.0	(6.2)	24.0	(6.9)	25.2	(7.4)	*P*<0.001
PUFA (g/d)	15.2	(6.0)	15.7	(5.7)	15.8	(5.9)	16.8	(6.8)	17.8	(6.7)	*P*<0.001	11.6	(5.1)	11.7	(4.2)	12.0	(4.1)	12.8	(4.3)	13.0	(4.6)	*P*<0.001
Saturated FA (g/d)	27.4	(9.9)	31.2	(11.2)	31.8	(10.1)	35.5	(11.8)	37.1	(12.1)	*P*<0.001	21.0	(8.8)	22.9	(8.4)	24.4	(8.0)	26.3	(9.3)	27.6	(10.0)	*P*<0.001
n (%)																						
Menopausal Status																						*P*=0.31
Pre-mp	-	-	-	-	-	-	-	-	-	-		104	(18.9)	75	(13.6)	78	(14.2)	75	(13.6)	82	(14.9)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		24	(4.3)	21	(3.8)	32	(5.8)	21	(3.8)	29	(5.3)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		93	(16.9)	89	(16.2)	86	(15.6)	89	(16.2)	91	(16.5)	
Post-mp	-	-	-	-	-	-	-	-	-	-		330	(59.9)	366	(66.4)	355	(64.4)	366	(66.4)	348	(63.3)	
HRT																						*P*=0.34
Current User	-	-	-	-	-	-	-	-	-	-		91	(16.5)	86	(15.6)	101	(18.3)	97	(17.6)	97	(17.6)	
Former User	-	-	-	-	-	-	-	-	-	-		69	(12.5)	53	(9.6)	57	(10.4)	68	(12.3)	77	(14.0)	
Never Used	-	-	-	-	-	-	-	-	-	-		391	(71.0)	412	(74.8)	393	(71.3)	386	(70.1)	376	(68.4)	
Smoking											*P*=0.042											*P*=0.028
Current smoker	37	(9.4)	52	(13.3)	47	(12.0)	56	(14.3)	46	(11.8)		51	(9.3)	62	(11.2)	62	(11.3)	85	(15.4)	83	(15.1)	
Former smoker	217	(55.4)	193	(49.4)	237	(60.5)	217	(55.5)	217	(55.5)		189	(34.3)	175	(31.8)	173	(31.4)	167	(30.3)	185	(33.6)	
Never smoked	138	(35.2)	146	(37.3)	108	(27.5)	118	(30.2)	128	(32.7)		311	(56.4)	314	(57.0)	316	(57.3)	299	(54.3)	282	(51.3)	
Physical activity											*P*=0.16											*P*=0.56
Inactive	123	(31.4)	137	(35.0)	122	(31.1)	109	(27.9)	123	(31.5)		178	(32.3)	188	(34.1)	183	(33.2)	169	(30.7)	189	(34.4)	
Mod. inactive	103	(26.3)	93	(23.8)	95	(24.2)	79	(20.2)	101	(25.8)		182	(33.0)	182	(33.0)	175	(31.8)	182	(33.0)	156	(28.4)	
Mod. active	87	(22.2)	88	(22.5)	84	(21.5)	94	(24.0)	83	(21.2)		113	(20.5)	114	(20.7)	104	(18.9)	117	(21.2)	129	(23.4)	
Active	79	(20.1)	73	(18.7)	91	(23.2)	109	(27.9)	84	(21.5)		78	(14.2)	67	(12.2)	89	(16.1)	83	(15.1)	76	(13.8)	
Family history*	11	(2.8)	12	(3.1)	10	(2.6)	11	(2.8)	14	(3.6)	*P*=0.93	27	(4.9)	36	(6.5)	32	(5.8)	24	(4.4)	35	(6.4)	*P*=0.45
Steroids	16	(4.1)	8	(2.1)	16	(4.1)	12	(3.1)	16	(4.1)	*P*=0.42	24	(4.4)	20	(3.6)	16	(2.9)	32	(5.8)	22	(4.0)	*P*=0.17
Calcium supp.	7	(1.8)	4	(1.0)	5	(1.3)	5	(1.3)	4	(1.0)	*P*=0.88	35	(6.4)	30	(5.4)	36	(6.5)	26	(4.7)	28	(5.1)	*P*=0.63
Vitamin D supp.	97	(24.7)	89	(22.8)	90	(23.0)	79	(20.2)	75	(19.2)	*P*=0.33	181	(32.9)	176	(31.9)	185	(33.6)	159	(28.9)	174	(31.6)	*P*=0.51

Abbreviations: Mp, menopausal; Family history of OP, family history of osteoporosis; Supp., supplements.

Values are means (standard deviations) or numbers (frequencies).

Table 7.5: Baseline characteristics of the 1957 men and 2754 women stratified by quintiles of the iron source ratio (% animal).

	Men											Women										
Ratio (% animal)	**Quintile 1 0.1 – 1.6**		**Quintile 2 1.7 – 2.2**		**Quintile 3 2.3 – 2.8**		**Quintile 4 2.9 – 3.7**		**Quintile 5 3.8 – 16.4**			**Quintile 1 0.0 – 11.5**		**Quintile 2 11.6 – 16.2**		**Quintile 3 16.3 – 20.6**		**Quintile 4 20.7 – 27.0**		**Quintile 5 27.1 – 85.0**		
	n = 392		***n = 391***		***n = 392***		***n = 391***		***n = 391***		***P*-trend**	***n = 551***		***n = 551***		***n = 551***		***n = 551***		***n = 550***		***P*-trend**
Mean (SD)																						
Age (years)	58.6	(9.9)	60.6	(9.5)	59.9	(9.4)	59.1	(9.1)	60.4	(9.7)	*P*=0.15	58.0	(9.8)	59.5	(9.4)	60.3	(9.6)	60.1	(9.0)	61.0	(9.7)	*P*<0.001
BMI (kg/m^2)	25.9	(3.2)	26.1	(3.0)	26.7	(3.3)	26.6	(3.1)	27.2	(3.7)	*P*<0.001	25.5	(4.3)	26.0	(4.0)	25.6	(4.0)	26.6	(4.7)	27.1	(4.4)	*P*<0.001
Plant iron (mg/d)	11.9	(4.9)	9.4	(3.1)	8.4	(2.8)	7.6	(2.2)	6.3	(2.2)	*P*<0.001	9.6	(3.8)	8.1	(2.9)	7.1	(2.2)	6.3	(2.0)	5.2	(2.0)	*P*<0.001
Animal iron (mg/d)	1.4	(0.6)	2.1	(0.6)	2.6	(0.8)	3.3	(0.9)	4.6	(1.9)	*P*<0.001	1.0	(0.5)	1.6	(0.5)	2.0	(0.6)	2.4	(0.7)	3.4	(1.6)	*P*<0.001
Total iron (mg/d)	15.2	(5.1)	13.6	(3.8)	12.8	(3.6)	12.6	(3.1)	12.5	(3.8)	*P*<0.001	12.2	(4.1)	11.3	(3.3)	10.7	(2.9)	10.2	(2.7)	9.9	(3.2)	*P*<0.001
Serum ferritin (ng/ml)	100.0	(78.3)	116.0	(81.0)	121.1	(88.7)	114.0	(78.9)	126.8	(95.6)	*P*=0.002	53.2	(44.1)	58.1	(42.6)	63.4	(50.3)	75.5	(60.6)	73.2	(59.4)	*P*<0.001
Energy (kcal/d)	2265	(506)	2258	(536)	2219	(490)	2272	(485)	2184	(539)	*P*=0.07	1700	(400)	1694	(359)	1727	(388)	1680	(380)	1617	(388)	*P*=0.001
Fat (g/d)	81.0	(24.3)	84.7	(27.3)	86.0	(25.1)	90.2	(25.7)	88.9	(27.4)	*P*<0.001	60.9	(20.8)	63.1	(19.1)	65.6	(20.5)	65.7	(20.4)	66.0	(20.4)	*P*<0.001
MUFA (g/d)	27.8	(8.8)	29.4	(9.5)	30.1	(9.0)	31.6	(9.2)	31.5	(9.7)	*P*<0.001	20.8	(7.5)	21.8	(6.9)	22.6	(7.3)	22.9	(7.1)	23.0	(7.1)	*P*<0.001
PUFA (g/d)	16.6	(6.2)	16.3	(6.9)	16.4	(6.0)	16.5	(6.0)	15.5	(6.3)	*P*=0.06	12.6	(5.0)	12.4	(4.3)	12.2	(4.4)	12.2	(4.5)	11.7	(4.3)	*P*=0.001
Saturated FA (g/d)	29.9	(10.8)	32.0	(11.8)	32.4	(11.1)	34.5	(11.3)	34.1	(12.2)	*P*<0.001	22.5	(9.0)	23.8	(8.5)	25.2	(9.4)	25.1	(9.4)	25.6	(9.6)	*P*<0.001
n (%)																						
Menopausal Status																						*P*=0.005
Pre-mp	-	-	-	-	-	-	-	-	-	-		107	(19.4)	81	(14.7)	83	(15.1)	65	(11.8)	78	(14.2)	
Peri-mp (<1 yr)	-	-	-	-	-	-	-	-	-	-		29	(5.3)	28	(5.1)	21	(3.8)	33	(6.0)	16	(2.9)	
Peri-mp (1-5 yrs)	-	-	-	-	-	-	-	-	-	-		100	(18.1)	96	(17.4)	79	(14.3)	90	(16.3)	83	(15.1)	
Post-mp	-	-	-	-	-	-	-	-	-	-		315	(57.2)	346	(62.8)	368	(66.8)	363	(65.9)	373	(67.8)	
HRT																						*P*=0.60
Current User	-	-	-	-	-	-	-	-	-	-		95	(17.2)	99	(18.0)	97	(17.6)	101	(18.3)	80	(14.5)	
Former User	-	-	-	-	-	-	-	-	-	-		66	(12.0)	54	(9.8)	64	(11.6)	66	(12.0)	74	(13.5)	
Never Used	-	-	-	-	-	-	-	-	-	-		390	(70.8)	398	(72.2)	390	(70.8)	384	(69.7)	396	(72.0)	
Smoking											*P*<0.001											*P*<0.001
Current smoker	23	(5.9)	44	(11.3)	53	(13.5)	53	(13.5)	65	(16.6)		48	(8.7)	42	(7.6)	53	(9.6)	89	(16.2)	111	(20.2)	
Former smoker	208	(53.0)	209	(53.4)	230	(58.7)	211	(54.0)	223	(57.0)		192	(34.9)	183	(33.2)	160	(29.1)	166	(30.1)	188	(34.2)	
Never smoked	161	(41.1)	138	(35.3)	109	(27.8)	127	(32.5)	103	(26.4)		311	(56.4)	326	(59.2)	338	(61.3)	296	(53.7)	251	(45.6)	
Physical activity											*P*=0.20											*P*=0.006
Inactive	105	(26.8)	120	(30.7)	127	(32.4)	121	(31.0)	141	(36.1)		171	(31.0)	163	(29.6)	176	(31.9)	175	(31.8)	222	(40.4)	
Mod. inactive	96	(24.5)	100	(25.6)	84	(21.4)	99	(25.3)	92	(23.5)		185	(33.6)	178	(32.3)	167	(30.3)	193	(35.0)	154	(28.0)	
Mod. active	94	(24.0)	98	(25.0)	88	(22.5)	78	(20.0)	78	(19.9)		126	(22.9)	114	(20.7)	121	(22.0)	107	(19.4)	109	(19.8)	
Active	97	(24.7)	73	(18.7)	93	(23.7)	93	(23.8)	80	(20.5)		69	(12.5)	96	(17.4)	87	(15.8)	76	(13.8)	65	(11.8)	
Family history*	12	(3.1)	11	(2.8)	11	(2.8)	12	(3.1)	12	(3.1)	*P*=1.00	33	(6.0)	29	(5.3)	27	(4.9)	31	(5.6)	34	(6.2)	*P*=0.89
Steroids	16	(4.1)	8	(2.0)	8	(2.0)	19	(4.9)	17	(4.4)	*P*=0.08	20	(3.6)	21	(3.8)	22	(4.0)	21	(3.8)	30	(5.5)	*P*=0.54
Calcium supp.	9	(2.3)	4	(1.0)	3	(0.8)	4	(1.0)	5	(1.3)	*P*=0.35	39	(7.1)	35	(6.4)	34	(6.2)	23	(4.2)	24	(4.4)	*P*=0.14
Vitamin D supp.	107	(27.3)	86	(22.0)	90	(23.0)	87	(22.3)	60	(15.4)	*P*=0.002	191	(34.7)	191	(34.7)	183	(33.2)	167	(30.3)	143	(26.0)	*P*=0.008

Abbreviations: Mp, menopausal; Family history of OP, family history of osteoporosis; Supp., supplements.

Values are means (standard deviations) or numbers (frequencies).

7.4.2 Associations between iron by food source and heel ultrasound

In univariate analyses in men, there were no correlations between iron intake from any sources or serum ferritin and measures of heel ultrasound **(Table 7.6)**. In contrast, in women, there were small but significant positive correlations between plant iron intake and both BUA and VOS (both r=0.08, *P*<0.05), and this reflected the relationship with total dietary iron intake which showed similar correlations with both measures of heel ultrasound (both r=0.08, *P*<0.05). Animal iron intake showed no relationship with the ultrasound measurements in women (r=0.00, P>0.05). However, the percentage contribution of animal iron towards total dietary iron intake showed a small and non-significant trend towards a negative relationship with BUA (r=-0.04) and VOS (r=-0.05, both *P*>0.05), which somewhat reflected the significant negative correlation between serum ferritin and BUA (r=-0.09) and VOS (r=-0.12, both *P*<0.05).

Table 7.6: Correlations between iron intake by food source with BUA and VOS.

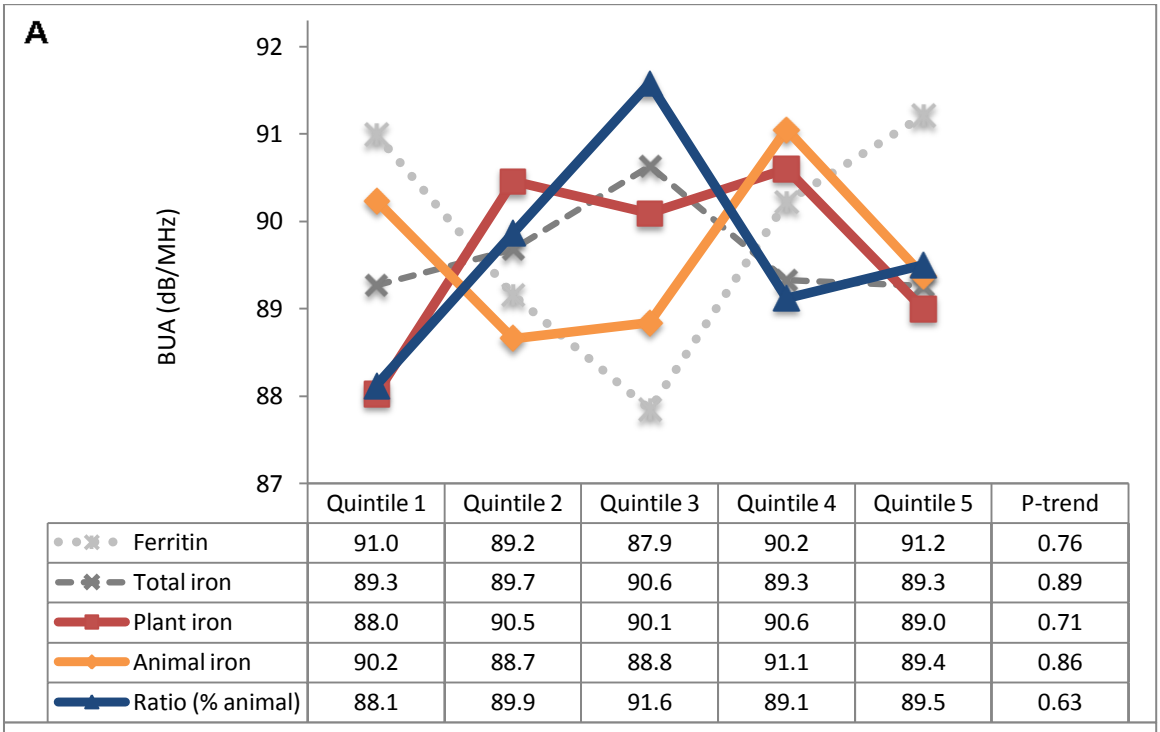
	Men		Women	
	BUA	VOS	BUA	VOS
Serum ferritin	0.02	0.01	-0.09*	-0.12*
Total dietary iron intake	-0.01	0.02	0.08*	0.08*
Plant iron intake	-0.01	0.03	0.08*	0.08*
Animal iron intake	0.00	-0.04	0.00	0.00
Ratio (% animal)	0.00	-0.06	-0.04	-0.05

*Pearson correlation coefficients were significant at *P<0.05.* For iron intake: *n*=968 men and *n*=1359 women; and for serum ferritin: *n*=682 men and *n*=910 women.

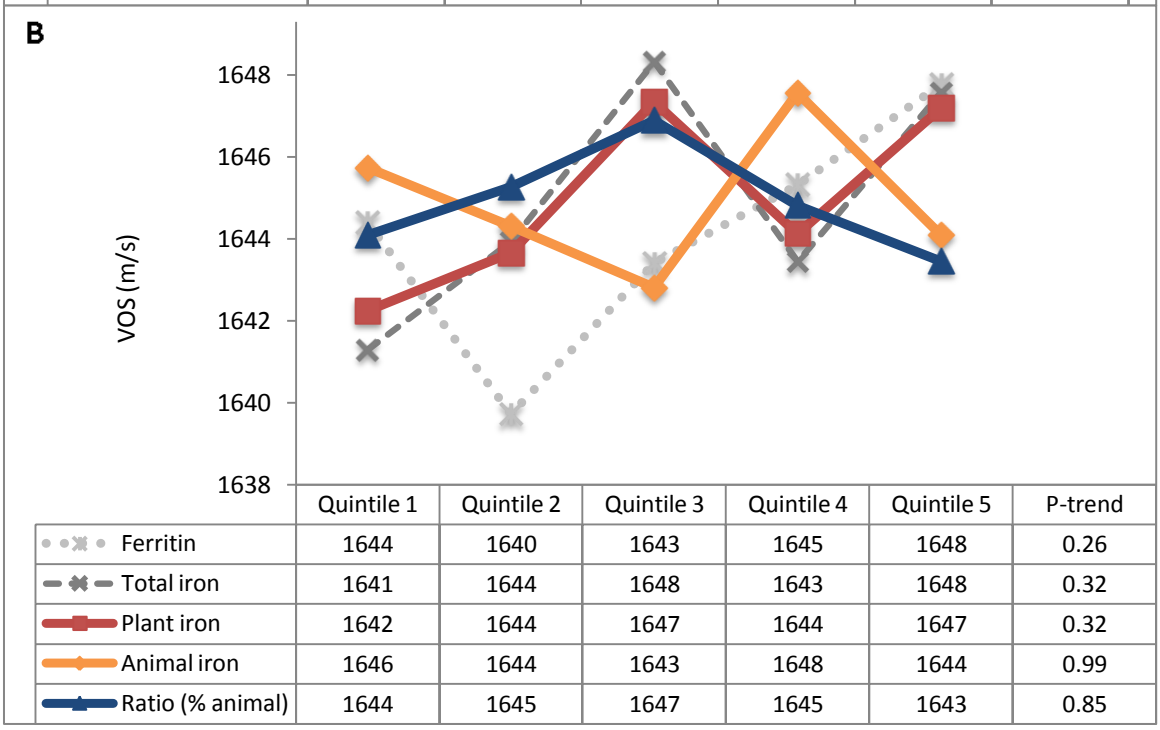
Associations between iron intake by food source and measures of heel ultrasound are shown in **Figure 7.3** for men and **Figure 7.4** for women. Briefly, higher plant iron intake was significantly associated with both parameters of ultrasound in women, but there were no associations in men. For comparison reasons, the associations with total dietary iron intake and serum ferritin concentrations, which were investigated in the previous chapter (Chapter 6, page 143), were also included in these graphs. In concordance with the findings from the univariate investigations in men, the results from the multivariate-adjusted linear regression analyses showed that quintiles of iron intake from any sources were not associated with measures of heel ultrasound. Interestingly, in men, the association between plant iron intake and VOS was almost identical to that of total dietary iron intake **(Figure 7.3B)**. Following the adjustment for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status, HRT, total energy intake, dietary calcium intake, calcium supplements and vitamin D supplements in women, there was a positive linear relationship between quintiles of plant iron intake and both BUA (β 0.79 dB/MHz per quintile, *P*-trend=0.010) and VOS (β 1.58 m/s per quintile, *P*-trend=0.038; **Figure 7.4**). These positive associations somewhat reflected those of total dietary iron intake and BUA (β 0.66 dB/MHz per quintile, *P*-trend=0.045), although the

associations with plant iron were stronger. Moreover, in women, there were significant differences in both BUA and VOS between the two upper quintiles of plant iron intake referent to quintile 1, with BUA being 5.8% higher for quintile 4 of plant iron (β 4.03 dB/MHz, *P*=0.002) and 4.5% higher for quintile 5 (β 3.16 dB/MHz, *P*=0.019), and VOS being 0.5% higher for quintile 4 (β 8.88 m/s, *P*=0.006) and 0.4% higher for quintile 5 (β 7.20 m/s, *P*=0.032). Interestingly, in women, associations between the animal iron ratio and heel ultrasound followed a similar pattern to that of serum ferritin. Although none of these associations were significant, there was a tendency for a negative linear association between BUA and the animal iron ratio (β -0.53 dB/MHz per quintile, *P*-trend=0.057) and between extreme quintiles of the latter (β -2.54 dB/MHz, *P*=0.042).

Figure 7.3: Associations in men between iron intake by food source with mean BUA **(A)** and VOS **(B)** in comparison to total dietary iron intake and serum ferritin.



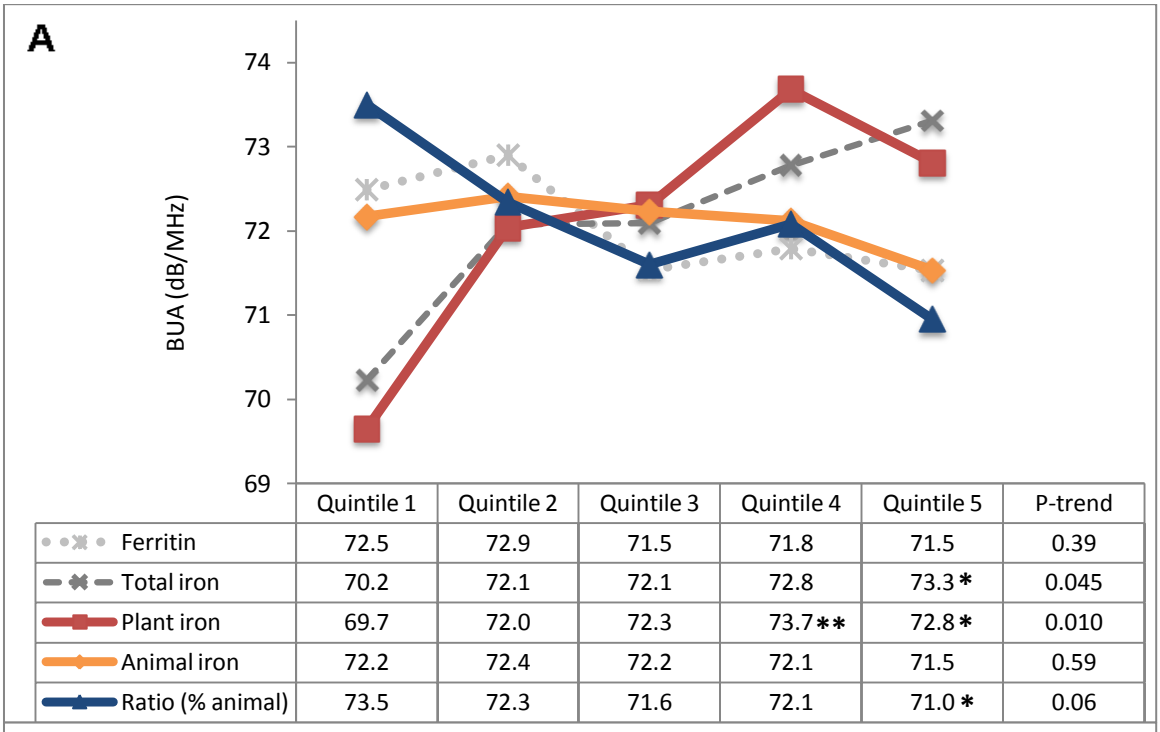
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Ferritin	91.0	89.2	87.9	90.2	91.2	0.76
Total iron	89.3	89.7	90.6	89.3	89.3	0.89
Plant iron	88.0	90.5	90.1	90.6	89.0	0.71
Animal iron	90.2	88.7	88.8	91.1	89.4	0.86
Ratio (% animal)	88.1	89.9	91.6	89.1	89.5	0.63



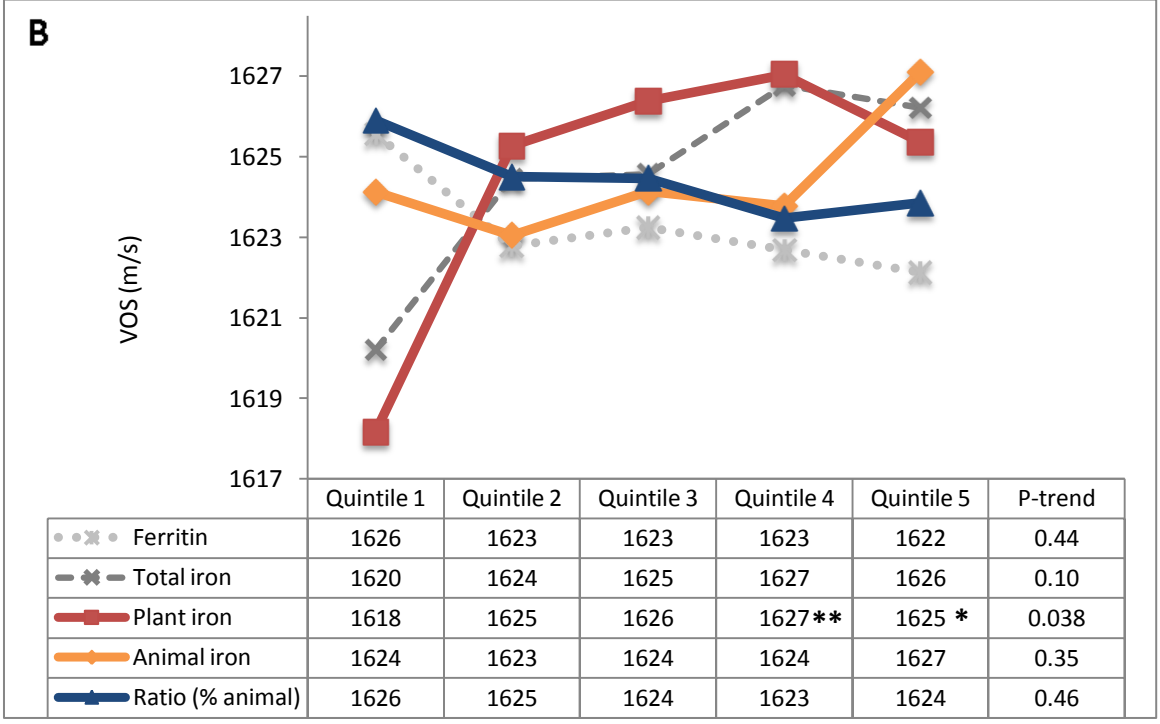
	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Ferritin	1644	1640	1643	1645	1648	0.26
Total iron	1641	1644	1648	1643	1648	0.32
Plant iron	1642	1644	1647	1644	1647	0.32
Animal iron	1646	1644	1643	1648	1644	0.99
Ratio (% animal)	1644	1645	1647	1645	1643	0.85

The mean iron intake for the lowest and highest quintiles respectively was 4.9±0.9 and 14.6±4.4 mg/d for plant iron, 1.2±0.4 and 4.9±1.2 mg/d for animal iron, and 9.2±3.1 and 35.4±6.8 % for the ratio. The standard error of the mean (SE) was 1.2-1.5 dB/MHz for BUA and 2.8-3.4 m/s for VOS. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. There were no significant differences between the two upper quintiles referent to quintile 1. n=968 for iron intake and n=682 for serum ferritin.

Figure 7.4: Associations in women between iron intake by food source with mean BUA **(A)** and VOS **(B)** in comparison to total dietary iron intake and serum ferritin.



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Ferritin	72.5	72.9	71.5	71.8	71.5	0.39
Total iron	70.2	72.1	72.1	72.8	73.3 *	0.045
Plant iron	69.7	72.0	72.3	73.7 **	72.8 *	0.010
Animal iron	72.2	72.4	72.2	72.1	71.5	0.59
Ratio (% animal)	73.5	72.3	71.6	72.1	71.0 *	0.06



	Quintile 1	Quintile 2	Quintile 3	Quintile 4	Quintile 5	P-trend
Ferritin	1626	1623	1623	1623	1622	0.44
Total iron	1620	1624	1625	1627	1626	0.10
Plant iron	1618	1625	1626	1627 **	1625 *	0.038
Animal iron	1624	1623	1624	1624	1627	0.35
Ratio (% animal)	1626	1625	1624	1623	1624	0.46

*The mean iron intake for the lowest and highest quintiles respectively was 4.2±0.8 and 12.2±2.3 mg/d for plant iron, 0.8±0.3 and 3.7±1.5 mg/d for animal iron, and 7.6±2.8 and 32.4±6.9 % for the ratio. The standard error of the mean (SE) was 0.9-1.1 dB/MHz for BUA and 2.1-2.7 m/s for VOS. The analysis used EPIC-Norfolk data from the second health check and was based on a multivariate-adjusted linear regression analysis. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05 and **P<0.01. n=1359 for iron intake and n=910 for serum ferritin.*

7.4.3 Associations between iron from different food sources and fracture risk

In the case-cohort sub-sample of EPIC-Norfolk participants, there were 112 hip fractures, 78 spine fractures and 70 wrist fractures in men, and 339 hip fractures, 124 spine fractures and 218 wrist fractures in women. In the case-cohort that investigated participants with a fracture at any of these three fracture sites combined (total fracture), there were 248 and 616 fractures in men and women, respectively. The results from the investigations of potential associations between iron intake by food source and fracture risk are discussed below. Briefly, iron intake from animal sources and the ratio of animal iron were significantly associated with higher hip fracture risk in men, whereas the former was significantly associated with lower spine fracture risk in women; and no associations were found for plant iron intake in either sex.

7.4.3.1 Iron intake by food source in fracture and non-fracture participants

Both iron intake from animal sources and the animal iron ratio did not differ between participants with and without a total fracture **(Table 7.7)**. However, women with a fracture had significantly lower iron intake from plant sources compared to those women who remained free from fractures over the median follow up of 12.6 years (7.0±3.1 *vs.* 7.3±3.0 mg/d, *P*=0.032). In men, plant iron intake did not differ between fracture and non-fracture participants.

Table 7.7: Iron intake by food source in subjects with and without a total fracture.

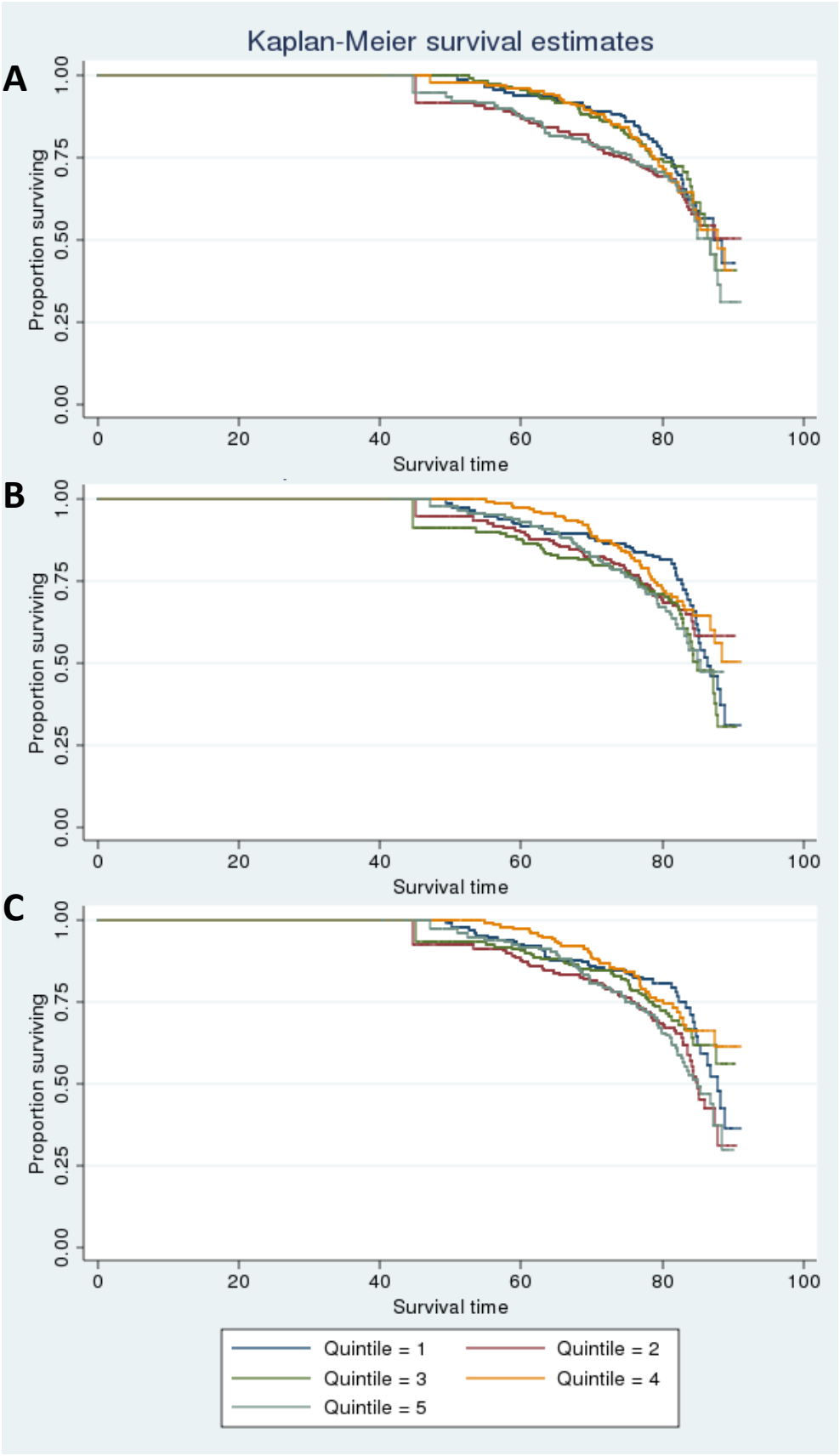
	Subjects without a fracture				Subjects with a fracture				
	n	**Mean**	**(SD)**	**[Range]**	***n***	**Mean**	**(SD)**	**[Range]**	***P***
Men									
Dietary iron intake (mg/d)	1709	13.3	(4.0)	[3.4; 42.2]	248	13.5	(4.5)	[5.7; 37.1]	0.39
Plant iron intake (mg/d)	1709	8.7	(3.7)	[1.2; 39.1]	248	8.7	(3.9)	[2.3; 29.1]	0.91
Animal iron intake (mg/d)	1709	2.8	(1.5)	[0.1; 16.4]	248	3.0	(1.8)	[0.1; 11.7]	0.09
Ratio (% animal)	1709	21.6	(10.1)	[0.8; 84.6]	248	22.3	(10.7)	[0.7; 62.9]	0.31
Women									
Dietary iron intake (mg/d)	2138	11.0	(3.4)	[1.9; 29.7]	616	10.5	(3.5)	[3.7; 27.0]	0.003
Plant iron intake (mg/d)	2138	7.3	(3.0)	[0.0; 24.9]	616	7.0	(3.1)	[1.05; 24.2]	0.032
Animal iron intake (mg/d)	2138	2.1	(1.2)	[0.0; 24.3]	616	2.0	(1.1)	[0.1; 9.9]	0.54
Ratio (% animal)	2138	19.5	(9.8)	[0.0; 85.0]	616	20.3	(10.2)	[0.7; 81.4]	0.11

7.4.3.2 Differences in hazard ratios of fracture risk by iron sources

In men, all Kaplan Meier plots showed that there was both overlap and cross-over between the five quintiles of iron intake, independent of the food source **(Figure 7.5)**. Nevertheless, the log-rank test for equality showed that total fracture incidence differed between quintiles of the animal iron ratio (*P*=0.030). In contrast to the previous investigations of total dietary iron intake and serum ferritin with fracture risk in men (Chapter 6, page 143), which were all non-significant, there were some significant associations with iron intake according to the food source **(Table 7.8)**. For example, higher animal iron intake was significantly associated with higher fracture risk at the hip (HR 1.22, 95%CI 1.04-1.42; *P*=0.012). Moreover, men in the upper quintiles of animal iron intake compared to those in quintile 1 had significantly higher hip fracture risk (quintile 4: HR 2.14, 95%CI 1.05-4.37, *P*=0.036; quintile 5: HR 2.29, 95%CI 1.11-4.73, *P*=0.025). In men, there was also a significant linear trend between a higher animal ratio and higher hip fracture risk (HR 1.20, 95%CI 1.03-1.40, *P*=0.021); and those men in the top quintile compared to the lowest quintile of the animal ratio had a significantly higher hip fracture risk (HR 2.61, 95%CI 1.25-5.45, *P*=0.011) and total fracture risk (HR 1.61, 95%CI 1.03-2.53, *P*=0.038). Interestingly, there was a trend for an inverse association between animal iron intake and wrist fractures, although was not significant (HR 0.83, 95%CI 0.69-1.01, *P*=0.059). Plant iron intake was not associated with fracture risk at any site in men.

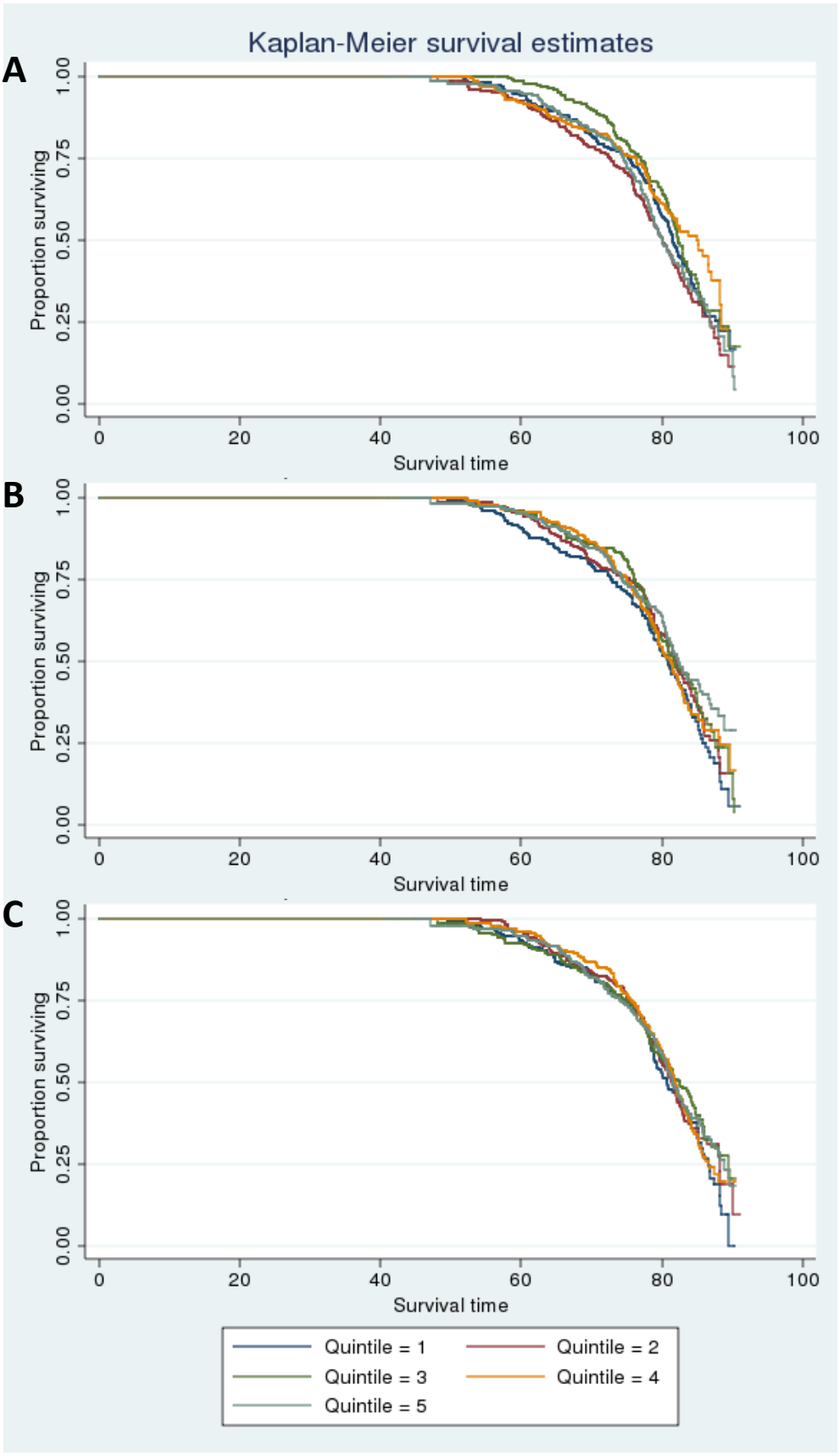
In women, there was both overlap and cross-over between the five quintiles of iron intake, independent of the food source, as shown by all Kaplan Meier plots **(Figure 7.6)**. Nevertheless, the log-rank test for equality showed that total fracture incidence differed between quintiles of plant iron intake (*P*=0.017). However, results from the calculation of Prentice-weighted Cox proportional HRs showed that both plant iron intake and the ratio of animal iron were not associated with fracture risk at any site in women **(Table 7.9)**. In contrast, women in the top compared to the lowest quintile of animal iron intake had a significantly lower spine fracture risk (HR 0.44, 95%CI 0.24-0.82, *P*=0.009). These results are comparable to the previous investigations in Chapter 7, where HRs for spine fracture risk were 0.41 (95%CI 0.21-0.79, *P*=0.008) for total dietary iron intake and 0.44 (95%CI 0.22-0.87, *P*=0.018) for serum ferritin. However, the previously established significant inverse associations between spine fracture risk and total dietary iron intake (HR 0.85, 95%CI 0.73-0.99, *P*-trend=0.041) and serum ferritin concentrations (HR 0.78, 95%CI 0.65-0.94, *P*-trend=0.009) were not significant in the present investigations of animal iron intake (HR 0.87, 95%CI 0.75-1.01, *P*=0.08).

Figure 7.5: Kaplan-Meier plot of total fractures by quintiles of iron intake by food source in 1957 men.



*Kaplan-Meier survival estimates for quintiles of iron intake from **(A)** plant sources, **(B)** animal sources and **(C)** the animal iron ratio. The quintile groups of the animal ratio differed significantly according to the log-rank test for equality (P=0.030), but there were no differences between the plant iron quintiles groups (P=0.91) and the animal iron quintile groups (P=0.38).*

Figure 7.6: Kaplan-Meier plot of total fractures by quintiles of iron intake by food source in 2754 women.



*Kaplan-Meier survival estimates for quintiles of iron intake from **(A)** plant sources, **(B)** animal sources and **(C)** the animal iron ratio. The quintile groups of plant iron intake differed significantly according to the log-rank test for equality (P=0.017), but there were no differences between the quintiles groups of animal iron intake (P=0.09) and those of the animal iron ratio (P=0.46).*

Table 7.8: Associations between iron intake by food source and fracture risk in men of the EPIC-Norfolk case-cohort in comparison to total dietary iron intake and serum ferritin.

		Quintile 1 *n = 392*	Quintile 2 *n = 391*		Quintile 3 *n = 392*		Quintile 4 *n = 391*		Quintile 5 *n = 391*		
	Iron intake	**HR (ref)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	**Serum ferritin**	1.00	0.68	(0.40-1.15)	0.78	(0.46-1.31)	0.58	(0.33-1.00)	0.88	(0.53-1.47)	*P*=0.51
(248 events)	**Total dietary iron**	1.00	0.72	(0.46-1.15)	0.90	(0.56-1.46)	0.97	(0.59-1.58)	1.12	(0.67-1.86)	*P*=0.36
	Plant iron	1.00	0.98	(0.63-1.53)	1.04	(0.65-1.65)	1.02	(0.61-1.69)	1.11	(0.68-1.82)	*P*=0.64
	Animal iron	1.00	1.02	(0.65-1.60)	1.11	(0.71-1.74)	0.99	(0.61-1.58)	1.27	(0.80-2.00)	*P*=0.41
	Ratio (% animal)	1.00	1.38	(0.88-2.15)	0.97	(0.61-1.56)	1.00	(0.62-1.59)	**1.61**	**(1.03-2.53)***	*P*=0.20
Hip fracture	**Serum ferritin**	1.00	0.88	(0.40-1.94)	1.09	(0.48-2.44)	0.69	(0.30-1.57)	0.91	(0.40-2.08)	*P*=0.64
(112 events)	**Total dietary iron**	1.00	0.80	(0.41-1.56)	1.00	(0.51-1.98)	1.17	(0.57-2.40)	0.92	(0.45-1.90)	*P*=0.80
	Plant iron	1.00	1.00	(0.54-1.84)	0.81	(0.40-1.67)	1.13	(0.57-2.26)	0.86	(0.42-1.78)	*P*=0.84
	Animal iron	1.00	1.45	(0.72-2.95)	1.70	(0.83-3.47)	**2.14**	**(1.05-4.37)***	**2.29**	**(1.11-4.73)***	***P*=0.012**
	Ratio (% animal)	1.00	1.85	(0.88-3.88)	1.19	(0.54-2.64)	1.66	(0.78-3.54)	**2.61**	**(1.25-5.45)***	***P*=0.021**
Spinal fracture	**Serum ferritin**	1.00	0.68	(0.29-1.64)	0.47	(0.16-1.31)	0.63	(0.25-1.58)	1.13	(0.50-2.56)	*P*=0.87
(78 events)	**Total dietary iron**	1.00	0.79	(0.33-1.88)	1.31	(0.58-2.94)	1.11	(0.44-2.83)	1.84	(0.77-4.39)	*P*=0.12
	Plant iron	1.00	1.04	(0.47-2.33)	1.41	(0.65-3.06)	1.28	(0.51-3.21)	1.54	(0.64-3.70)	*P*=0.30
	Animal iron	1.00	1.10	(0.51-2.39)	1.33	(0.64-2.75)	0.79	(0.33-1.86)	1.50	(0.70-3.19)	*P*=0.52
	Ratio (% animal)	1.00	1.44	(0.69-3.02)	1.09	(0.49-2.43)	1.07	(0.48-2.35)	1.49	(0.68-3.24)	*P*=0.61
Wrist fracture	**Serum ferritin**	1.00	0.47	(0.18-1.25)	0.72	(0.32-1.61)	0.57	(0.23-1.40)	0.56	(0.22-1.39)	*P*=0.31
(70 events)	**Total dietary iron**	1.00	0.53	(0.23-1.26)	0.73	(0.31-1.73)	0.65	(0.29-1.45)	0.98	(0.42-2.29)	*P*=0.75
	Plant iron	1.00	0.97	(0.42-2.25)	1.11	(0.50-2.44)	0.62	(0.25-1.50)	1.18	(0.53-2.62)	*P*=0.97
	Animal iron	1.00	0.72	(0.35-1.49)	0.58	(0.27-1.25)	0.46	(0.21-1.02)	0.52	(0.24-1.12)	*P*=0.06
	Ratio (% animal)	1.00	0.81	(0.38-1.72)	0.71	(0.34-1.45)	0.52	(0.24-1.13)	0.78	(0.37-1.64)	*P*=0.28

*Values are adjusted Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. Iron intake from plant and animal sources was in mg/d, and the animal ratio in %. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05. n 1957 for total fracture, n 1842 for hip fracture, n 1808 for spine fracture, n 1806 for wrist fracture.*

Table 7.9: Associations between iron intake by food source and fracture risk in women of the EPIC-Norfolk case-cohort in comparison to total dietary iron intake and serum ferritin.

		Quintile 1 *n = 551*	Quintile 2 *n = 551*		Quintile 3 *n = 551*		Quintile 4 *n = 551*		Quintile 5 *n = 550*		
	Iron intake	**HR (ref)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	**HR**	**(95%CI)**	***P*-trend**
Total fracture	**Serum ferritin**	1.00	0.72	(0.48-1.08)	0.62	(0.42-0.93)	0.71	(0.48-1.05)	0.73	(0.49-1.08)	*P*=0.23
(616 events)	**Total dietary iron**	1.00	0.95	(0.69-1.30)	1.20	(0.87-1.66)	**0.65**	**(0.47-0.92)***	1.12	(0.78-1.62)	*P*=0.61
	Plant iron	1.00	1.23	(0.91-1.66)	0.92	(0.67-1.27)	0.85	(0.61-1.17)	1.15	(0.83-1.59)	*P*=0.77
	Animal iron	1.00	0.84	(0.62-1.14)	0.82	(0.60-1.11)	0.91	(0.65-1.26)	0.75	(0.53-1.04)	*P*=0.19
	Ratio (% animal)	1.00	0.93	(0.68-1.28)	0.82	(0.60-1.12)	0.92	(0.67-1.27)	0.85	(0.61-1.18)	*P*=0.40
Hip fracture	**Serum ferritin**	1.00	0.68	(0.39-1.19)	0.66	(0.38-1.14)	0.93	(0.55-1.55)	0.93	(0.57-1.54)	*P*=0.57
(339 events)	**Total dietary iron**	1.00	1.21	(0.81-1.81)	1.44	(0.95-2.19)	0.68	(0.44-1.07)	1.57	(0.98-2.52)	*P*=0.65
	Plant iron	1.00	1.26	(0.85-1.85)	1.11	(0.74-1.66)	0.75	(0.49-1.16)	1.32	(0.87-2.01)	*P*=0.91
	Animal iron	1.00	0.86	(0.58-1.28)	0.82	(0.54-1.23)	1.04	(0.68-1.59)	0.81	(0.52-1.26)	*P*=0.65
	Ratio (% animal)	1.00	0.69	(0.45-1.04)	0.64	(0.42-0.97)	0.83	(0.55-1.26)	0.76	(0.50-1.17)	*P*=0.52
Spinal fracture	**Serum ferritin**	1.00	0.62	(0.32-1.20)	0.38	(0.18-0.81)	**0.30**	**(0.14-0.64)****	**0.44**	**(0.22-0.87)***	***P*=0.009**
(124 events)	**Total dietary iron**	1.00	0.33	(0.18-0.62)	0.67	(0.37-1.20)	**0.51**	**(0.29-0.92)***	**0.41**	**(0.21-0.79)****	***P*=0.041**
	Plant iron	1.00	1.43	(0.85-2.41)	0.76	(0.41-1.39)	0.94	(0.51-1.74)	0.83	(0.46-1.52)	*P*=0.25
	Animal iron	1.00	0.52	(0.29-0.92)	0.50	(0.28-0.90)	0.73	(0.41-1.28)	**0.44**	**(0.24-0.82)****	*P*=0.08
	Ratio (% animal)	1.00	1.33	(0.73-2.41)	1.03	(0.56-1.90)	0.68	(0.35-1.31)	0.89	(0.48-1.65)	*P*=0.21
Wrist fracture	**Serum ferritin**	1.00	0.74	(0.42-1.31)	0.62	(0.35-1.09)	0.68	(0.39-1.20)	0.74	(0.42-1.30)	*P*=0.37
(218 events)	**Total dietary iron**	1.00	0.99	(0.63-1.57)	1.16	(0.73-1.83)	0.74	(0.45-1.22)	0.95	(0.56-1.62)	*P*=0.48
	Plant iron	1.00	0.88	(0.58-1.34)	0.68	(0.43-1.08)	0.71	(0.45-1.13)	0.89	(0.56-1.40)	*P*=0.38
	Animal iron	1.00	1.16	(0.74-1.81)	1.19	(0.75-1.87)	1.09	(0.67-1.77)	0.97	(0.58-1.63)	*P*=0.84
	Ratio (% animal)	1.00	1.12	(0.70-1.80)	1.06	(0.66-1.70)	1.27	(0.78-2.04)	1.08	(0.66-1.78)	*P*=0.62

*Values are adjusted Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). The analysis used data from the first health check. Iron intake from plant and animal sources was in mg/d, and the animal ratio in %. Differences between the two upper quintiles referent to quintile 1 were significant at *P<0.05 and **P<0.01. n 2754 for total fracture, n 2525 for hip fracture, n 2334 for spine fracture, n 2409 for wrist fracture.*

7.5 Discussion

To the best of my knowledge, these data are the first to investigate potential associations between dietary iron intake from different food sources with measures of bone health in a cross-sectional study, and with the risk of fractures in a prospective study of a large sample of British men and women. Following multivariate adjustment, the results from the cross-sectional study showed that higher plant iron intake was significantly associated with higher heel ultrasound measurements in women only, and no relationship with animal iron intake or the ratio of animal iron to total dietary iron intake was found in either sex. The results from the prospective investigations were contradictory and showed that higher animal iron intake was significantly associated with reduced spine fracture risk in women but with increased hip fracture risk in men, and there were no associations between plant iron intake and fracture risk at any site in either sex.

7.5.1 Heel ultrasound

In our novel cross-sectional study, dietary iron intake was not associated with heel ultrasound in men, and this was independent of the food source. In contrast, in women, plant iron intake correlated significantly with both BUA and VOS in women (r=0.08), possibly due to women in this cohort having significantly higher dietary intakes of fruit and vegetables than men (284±169 *vs.* 250±164 g/d). Moreover, there was a significant linear relationship between quintiles of plant iron intake and both measures of heel ultrasound in women, and plant iron intakes of 7.7-9.5 mg/d and 9.5-20.7 mg/d compared to 1.8-5.2 mg/d were significantly associated with 5.8% and 4.5% higher BUA and 0.5% and 0.4% higher VOS in women, respectively. Plant-based iron is predominantly found in pulses, whole grains and some green leafy vegetables including spinach and broccoli [(145)]. The differences in mean plant iron intake between the two upper quintiles in women were 4 mg/d for quintile 4 and 8 mg/d for quintile 5. These higher plant iron intakes can be achieved through the usual diet, although particular attention should be paid to consuming a variety of iron-rich foods. For example, three tablespoons of green or brown lentils contain approximately 4mg of plant iron; whereas ten cashews as snack foods combined with four tablespoons of green or brown lentils and four spears of broccoli as part of a main meal would need to be consumed additionally to the reach the levels of intake for quintile 5 [(145, 350)]. Despite the significant relationship between plant-based iron and measures of bone heath in women in the present study, there were no associations with animal iron intake. However, there was a tendency for a negative relationship between a higher ratio of animal iron to total dietary iron intake and BUA in women, although this was not significant. The cross-sectional findings of a positive association between plant-based iron intake and measures of heel ultrasound in women potentially reflect the important role of iron in bone health. It is well documented that iron plays

a crucial role as a cofactor in the hydroxylation reactions within collagen fibres [8, 10], which increases overall collagen strength [136], as well as in the synthesis of vitamin D [172, 383], an important mediator in calcium absorption [125]. To date, it is not known whether the different sources of iron intake influence the underlying mechanisms in bone health differently.

To the best of my knowledge, no previous studies have investigated a potential difference between iron intakes from different food sources in association with bone health. Thus, the present findings of a significant positive association between plant iron intake and measures of heel ultrasound in women are completely novel. The effect sizes of 4.5-5.8% higher BUA and 0.4-0.5% higher VOS between upper and lower quintiles of plant iron intake in women in our study were comparable to the findings from a US study which reported effect sizes of 4-14% higher BMD depending on the bone site between extreme quartiles of total dietary iron intake [181]. A direct comparison with previous studies of the absolute values for the effect sizes was not possible, as no published observational study has used measures of heel ultrasound. However, in the previous chapter (Chapter 6, page 143), we investigated the cross-sectional association between total dietary iron intake and heel ultrasound in EPIC-Norfolk men and women, and our results showed that the highest compared to the lowest quintile of total dietary iron intake was significantly associated with 4.4% higher BUA in women. Interestingly, in women, the relationship between plant iron intake and heel ultrasound in the present chapter somewhat reflected that of total dietary iron intake in the previous chapter, but effect sizes with BUA were greater for plant iron intake (4.5-5.8%) than for total dietary iron intake (4.4%). This may suggest that, in women, total dietary iron intake is more reflective of iron intake from plant sources than from animal sources. In fact, there is evidence to show that the contribution of plant-based iron towards total dietary iron intake is greater than that of animal iron [193]. In the previous chapter, we also investigated the cross-sectional relationship between serum ferritin concentrations and heel ultrasound, but there were no significant associations in either sex. In contrast to iron intake, the relationship between the animal iron ratio and heel ultrasound in women followed a similar pattern to that of serum ferritin. Therefore, serum ferritin concentrations may be more indicative of a higher percentage contribution of animal iron to total dietary iron intake than of plant-based iron in women. A potential reason for this may be that animal iron is a greater contributor towards the body's iron pool than plant iron as it is much better absorbed (15-40% *vs.* 1-15%) [188-192]. Animal iron is more readily bioavailable and its absorption is not affected by factors which are known to inhibit plant iron absorption including the presence of absorption inhibitors including phytates and polyphenols or absorption enhancers including vitamin C [358, 359, 415]. In contrast to women, these relationships were less clear in men, and this may in part explain the non-significant associations between iron intake independent of the food source and heel ultrasound in men in the present study. For example, both plant-based iron and the animal

iron ratio followed a similar pattern to total dietary iron intake, but none of the iron food sources appeared to be reflective of serum ferritin concentrations in men.

Another potential explanation for the present sex-specific results may be that the contribution of animal and plant iron towards total dietary iron intake differs between men and women. For example, in the present study, men compared to women had a significantly higher ratio of the contribution of animal iron towards total dietary iron intake (21.7±10.2% *vs.* 19.7±9.9%, *P*<0.001). Our findings are in agreement with data from the NDNS, undertaken at the time of recruitment of EPIC-Norfolk participants, which showed that haem iron, which is only found in animal-based food sources, accounted for 6.6% and 6.2% of total dietary iron intake in older men and women, respectively [(223)]. This may suggest that men tend to have a higher intake of the more bioavailable haem iron from animal foods; whereas women tend to have a higher consumption of the less well absorbed non-haem iron from mainly plant-based food sources. To date, the potential consequences of this for bone health are not known. However, a higher intake of animal-based haem iron in men may be indicative of a less healthy dietary pattern characterised by a high consumption of animal foods such as red meats at the expense of plant-based foods such as fruit and vegetables. In contrast to men, a higher intake of plant iron in women may be indicative of a healthier dietary pattern characterised by a high consumption of fruit and vegetables. In previous epidemiological studies, higher dietary intakes of fruit and vegetables were associated with higher BMD [(90, 131, 416)], and this may partly explain the present sex-specific findings of a positive association between plant iron intake and heel ultrasound in women.

In conclusion, the present cross-sectional investigations found significant associations between higher plant iron intake and higher BUA in women only, but there were no associations with animal iron intake and the ratio of animal iron as a percentage of total dietary iron intake in either sex. These findings are completely novel, and thus more epidemiological studies are needed which will investigate potential associations between iron intake from different food sources and measures of bone health in other populations.

7.5.2 Fracture risk

In our novel prospective study, there were no associations between quintiles of iron intake from plant-based sources and fracture risk at any site in either sex, although women with a fracture had significantly lower iron intake from plant sources at baseline (7.0±3.1 mg/d) compared to those women who remained free from fractures over the median follow up of 12.6 years (7.3±3.0 mg/d). In contrast, the results for animal iron intake were sex-specific, with a significant inverse association with spine fracture risk in women and a significant positive association with hip fracture risk in men. For example, women with the highest animal iron intakes (2.8-24.3 mg/d) compared to those with the lowest intakes (0-1.2 mg/d) had a 56% lower spine fracture

risk. In contrast to women, there was a significant linear trend between higher animal iron intake and higher hip fracture risk in men. Moreover, men with animal iron intakes of 2.8-3.7 mg/d and 3.7-16.4 mg/d compared to those with the lowest intakes (0.1-1.7 mg/d) had significantly higher hip fracture risk (HR 2.14, 95%CI 1.05-4.37; and HR 2.29, 95%CI 1.11-4.73, respectively). The ratio of animal iron to total dietary iron intake showed similar results to the crude animal iron intake in men, in that there was a significant linear trend between a higher animal iron ratio and higher hip fracture risk. Moreover, the hazard ratios for hip fracture risk (HR 2.61, 95%CI 1.25-5.45) and for total fracture risk (HR 1.61, 95%CI 1.03-2.53) were also significantly higher in men with an animal iron ratio of 29.2-84.6% (mean: 36.9%) compared to 0.7-13.6% (mean: 9.2%). To the best of my knowledge, no previous epidemiological studies have investigated potential associations between iron intake from different food sources and fracture risk, and hence the present results are novel findings.

The reason for the present sex-specific findings for animal iron intake may partly be related to differences in the dietary pattern and absorption mechanisms of iron. As previously discussed, a higher intake of animal-based haem iron in men may be indicative of a less healthy dietary pattern characterised by a high consumption of animal foods such as red meats at the expense of plant-based foods such as fruit and vegetables. In previous epidemiological studies, lower dietary intakes of fruit and vegetables were associated with lower BMD [90, 131, 416], and this may explain why hip fracture risk was increased in men with higher animal iron intake or a higher ratio of animal iron. In women, a higher intake of animal iron may be indicative of better iron status, as animal-based haem iron is more bioavailable and thus better absorbed than plant-based non-haem iron (15-40% *vs.* 1-15%) [188-192]. In fact, in the present study, women in quintile 5 compared to quintile 1 of animal iron intake had significantly higher total dietary iron intake (12.5±3.5 *vs.* 9.7±3.3 mg/d) and serum ferritin concentrations (72.0±57.9 *vs.* 55.4 ng/ml). Therefore, the significant reduction in spine fracture risk in women with higher animal iron intakes may be a reflection of the higher bioavailability of animal iron which may be associated with more adequate iron stores.

Interestingly, the present results of an inverse association between animal iron intake and spine fracture risk in women (HR 0.44, 95%CI 0.24-0.82) reflected the findings from our previous chapter (Chapter 6), which also showed significant spine fracture risk reductions for total dietary iron intake (HR 0.41, 95%CI 0.21-0.79) and for serum ferritin concentrations (HR 0.44, 95%CI 0.22-0.87). In contrast to women, the present results in men of a significant increase in hip fracture risk with higher animal iron intake did not reflect the findings from the previous chapter, which did not find an association with total dietary iron intake and serum ferritin in men. This may suggest that investigations of total dietary iron intake and markers of iron status in association with bone health may only provide limited insight into this relationship, and

differences in iron food sources should be taken into account as these provide additional information on the bioavailability of iron.

In conclusion, the present prospective investigations found significant associations between higher animal iron intake and reduced spine fracture risk in women but increased hip fracture risk in men, but there were no associations between plant iron intake and fracture risk at any site in either sex. These findings are completely novel, and thus more epidemiological studies are needed which will investigate potential associations between iron intake from different food sources and fracture risk in other populations.

7.5.3 Strengths and limitations

To the best of my knowledge, no previous epidemiological studies have explored the role of iron intake from animal and plant food sources in bone health, and thus the present cross-sectional and prospective investigations are completely novel. Previous studies have only explored the role of dietary iron in bone health independent of the food source [142, 181, 186, 405-407]. However, the intake of iron from foods provides no information on its bioavailability, which differs between the haem and non-haem forms of iron [188-192, 358, 359, 415], thereby potentially affecting the underlying mechanisms differently. Our study comprised up to 4711 men and women aged 39-79 years from the EPIC-Norfolk cohort and showed i) a significant positive association between plant iron intake and heel ultrasound in women and ii) a significant decrease in spine fracture risk in women but a significant increase in hip fracture risk in men with higher animal iron intakes. Our novel findings may have important implications for informing future guidelines on bone health, suggesting that the food source of iron in addition to an adequate dietary iron intake may play an important role in osteoporosis and fracture prevention.

Although the present study had a robust study design, there were also limitations. The cross-sectional study design of the bone density analyses only examined relations between diet and bone density for a single point in time. The positive associations reported in women suggest that there was a relation between plant iron intakes and heel ultrasound, but conclusions about the influence of plant iron on bone health cannot be drawn. Similarly, the prospective study design of the fracture analyses was limited by the inability to identify possible secular changes in animal and plant iron intakes over the follow-up period and subsequent exposure misclassification, as data were only available from the 7-day food diaries taken at baseline. Moreover, the fracture data had been obtained from hospital admissions which are most likely underestimated for spine fractures due to a large absence in their clinical attention and radiologic detection [168, 293, 294]. This may have reduced the power of the present study to detect the associations between animal and plant iron intake and spine fracture risk. Another limitation was related to the derivation of iron intake by food source in the present dataset. Following the initial coding of all food items in the EPIC-Norfolk study, the present dataset contained 21

categories of food sources which were based on dietary iron intakes. However, some categories contained foods of mixed animal and plant iron sources, for example “savoury pies and quiches” and “sandwich fillings with vegetables and egg or cheese”. These foods had an unknown contribution of plant and animal iron towards the total iron food content and could not be included in the present food source-specific iron analyses. Therefore, the dietary intakes of both plant and animal iron in the present dataset are incomplete, although we do not know the extent to which they were underestimated and whether this would affect our findings. Although multivariate adjustment models were applied in the analyses, a number of other relevant confounders previously associated with bone health, including sunlight exposure [295], were not measured as part of the EPIC-Norfolk study. Moreover, we could not account for factors which are known to affect non-haem iron absorption, including the iron content of a meal, an individual’s iron status and the presence of absorption inhibitors including phytic acid and polyphenols, and absorption enhancers including the reducing agent vitamin C [358, 359, 415]. These factors are specific to the absorption of non-haem iron and do not affect that of haem iron. Furthermore, residual confounding may have occurred despite the adjustment for covariates and may have resulted in bias in exposure effect estimates.

7.6 Conclusion

The present cross-sectional investigations in EPIC-Norfolk participants found that higher plant iron intakes were significantly associated with up to 5.8% and 0.5% higher BUA and VOS respectively in women only. These differences in bone density between women with low and high iron intakes from plant-based sources may have important implications for the development of fractures in the long term, although in our prospective investigations, we did not find an association between plant iron intake and fracture risk in either sex. However, higher animal iron intakes were significantly associated with a spine fracture hazard ratio of 0.44 in women, but a hip fracture hazard ratio of 2.29 in men, possibly due to sex-specific differences in habitual dietary intakes. These data are completely novel as previous studies have only investigated iron intake independent of the food source, but our findings suggest that the different food sources of iron intake may need to be taken into consideration when studying the role of iron in bone health as these provide additional information on the bioavailability of iron. Therefore, future epidemiological studies should investigate potential associations between iron intake from different food sources and bone health in other populations. These studies will be crucial for confirming the present sex-specific findings of a positive association between plant-based iron and bone density in women, and a potential role of animal iron in reducing spine fracture risk in women but increasing hip fracture risk in men.

CHAPTER 8

MEASUREMENT ERROR IN DIET-DISEASE RELATIONSHIPS

An investigation into how the combination of different dietary assessments of vitamin C affects its association with bone health

8.1 Abstract

Dietary assessment methods inaccurately reflect estimates of habitual food intake in populations due to measurement errors associated with their use, although biomarkers of dietary intake have other sources of error. Thus, establishing population intakes is associated with errors which may attenuate diet-disease relationships. Combining measures of dietary intake with biomarkers may be a useful approach for limiting the effects of such measurement error, but no previous studies have investigated this in bone health. Therefore, this chapter aimed to explore whether the addition of a biomarker to an intake estimate improves the detection and strength of the diet-disease association compared to using single measures at the example of vitamin C and bone health. The results showed that combining measures of vitamin C intake and plasma status strengthened the positive associations with heel ultrasound and inverse associations with fracture risk in men, but not in women. Our findings are completely novel. We suggest that more epidemiological studies investigate the concept of using a combination of dietary assessment methods in association with other common chronic disease risks.

8.2 Introduction

This chapter discusses the issue of bias and measurement error which naturally arise from estimating dietary intakes in epidemiological studies, and its effects on diet-disease associations. Assuming there is a positive relationship between vitamin C and bone health, the aim was to assess whether the addition of a biomarker to an intake estimate improves the detection and strength of the diet-disease association.

Epidemiological studies investigate the prevalence of an exposure, a disease rate or health outcome, or most commonly the association between these parameters, in a population. The overall aim of these studies is to detect any associations as well as their direction and strengths as accurately as possible. This relies on the use of measurements which reflect the true rather than estimated exposure and health outcome. However, as epidemiological studies are undertaken in humans and are subject to practical and ethical constraints, the introduction of bias and errors is unavoidable [(199)]. For example, errors associated with the measurement of exposure and outcome variables are common, and those are referred to as measurement errors. Exposure measurement errors may reduce the statistical power to detect any associations as they shift the estimated relative risk towards no effect, thus underestimating potential risk associations [(200)].

A common exposure assessed in relation to disease outcome is diet, and measuring dietary intake at the individual, household and national level introduces measurement error due to reporting bias and daily variations in dietary intake [(194, 198)]. It is possible to explore the relationship between true and observed exposure of diet from a validation study, which is useful for determining the extent to which observations differ from the truth, i.e. the extent to which estimated food intake differs from what was actually consumed, absorbed and utilised by the body [(199)]. To determine the accuracy of a chosen dietary assessment method, the comparison against a gold standard method is desirable, as those are independent of reporting bias arising from self-report methods [(198)]. In nutritional epidemiology, the measures considered to be most independent of dietary intake are biological markers, and hence those are most often used in validation studies. Agreement between the intake measure and the biological reference measure can indicate the usefulness of the former to estimate dietary intake. In validation studies, both the validity and the reproducibility of the intake measure are assessed. Validity is the ability of a measurement to measure what it purports to measure. For this, the agreement between the measure to be assessed and an absolute standard is evaluated. In nutritional epidemiology, however, all dietary assessment methods are prone to bias and no true reference measure exists. Hence, the test measure is compared to what is believed to be a more accurate measure of food and nutrient intake, assessing the “relative” validity of the dietary assessment methods. Reproducibility is the ability of a measurement to produce the same results when used

repeatedly in the same setting. However, in nutritional epidemiology, any one setting cannot be replicated as individual food intake varies by day, week and season. The variation in observations may be due to the actual daily variability of an individual's food consumption or biases associated with the chosen method of dietary assessment.

As previously discussed, the presence of exposure measurement error may reduce the statistical power to detect any diet-disease associations [200]. However, the adjustment for measurement error may limit the underestimation of the relative risk, although this does not compensate for the loss of statistical power [417]. Additional measurement errors, which occur during the measurement of confounders in multivariate models, increase the complexity of this issue, and this may result in over- or underestimation of relative risks by any magnitude [417]. Hence, the adjustment for measurement error is advised and numerous approaches have been suggested over the last few decades [418-425]. For example, a linear regression calibration includes a validation study which uses reference instruments such as biomarkers to relate true and observed exposure of the covariates [426]. One such study investigated the association between protein intake and incident frailty before and after correcting for measurement error [427]. They developed regression calibration equations of a biomarker (urinary nitrogen) and applied those to the FFQ estimates of protein intake. The results showed that protein intake was significantly inversely associated with incident frailty, and the use of the calibration method greatly improved the strength of the association by almost doubling the odds ratios. Another approach is that of combining different dietary intake measurements [206, 418], which has been suggested to increase the statistical power to detect the diet-disease association [205]. For example, one study investigated dietary lutein plus zeaxanthin, estimated from a FFQ and from serum concentrations of the carotenoids' *trans* isomers, in association with the risk of nuclear cataracts and nuclear sclerosis [206]. They ranked participants according to the mean dietary intakes and serum concentrations, before combining these ranks. The results showed that the association was stronger when using the biomarker compared to the intake estimate in the nuclear cataract investigations, whereas the associations were similar in the nuclear sclerosis analyses. Moreover, for both disease outcomes, the combination of the intake measure with the biomarker resulted in slightly improved odds ratios compared to using single measures. For example, the odds for nuclear cataract were 0.77 (95%CI 0.57-1.02) for the FFQ estimate, 0.69 (95%CI 0.51-0.94) for serum concentrations and 0.66 (95%CI 0.48-0.91) for the combination of the dietary intake with the biomarker. The results of this study may suggest that combining measures of dietary intake with a biomarker may be superior to using single measures in detecting the diet-disease relationship. To the best of my knowledge, only a limited number of studies have used the approach [206] and no such studies have investigated bone health. Therefore, this chapter will explore this approach using the association between dietary intakes and nutrient status of vitamin C with indicators of bone health as an example, due to the

availability of data in the EPIC-Norfolk study (vitamin C intake estimated from 7dDD and FFQ, plasma vitamin C, heel ultrasound, DXA and fractures) and due to the moderate correlation between vitamin C intake and blood measures reported in previous studies (r=0.20-0.55) [202, 303, 428-432]. The next sections will discuss vitamin C intake and blood status as exposure variables and bone health as an outcome variable.

8.2.1 Vitamin C intake and status as exposure variables

As discussed in detail in Chapter 1 (pages 34-36), there are a range of methods used in nutritional epidemiology to quantitatively assess food and nutrient intakes on the individual and population level [195]. All written dietary assessment methods and biomarkers are inaccurate estimates of the habitual dietary intake, and thus the choice of assessment usually depends on the sample size of the study population, the practicality of the assessment and evaluation of intake and the subsequent costs involved. The most commonly used methods are the 16-day weighed record, 7dDD, FFQ, 24hR, as well as biological markers of dietary intake. They mainly differ in the type of intake they assess (habitual or recent intake), the accuracy of intake assessment and their practicability.

8.2.1.1 Methods assessing dietary intakes of vitamin C

Dietary intakes of vitamin C may be assessed using any of the written methods above. Assessment of vitamin C intake from 7dDDs has been shown to yield the most comparable intake data with weighed records (r=0.70) [202, 204], the assessment method most commonly used in dietary validation studies, compared to FFQs and 24-h recalls (both r=0.54). In contrast, FFQs have consistently been shown to overestimate intakes of fruits and vegetables and consequently intakes of vitamin C [201-204]; whereas 24-hour recalls tend to underestimate vitamin C intakes due to the inability to assess seasonal and day-to-day variation [202, 204]. Moreover, the repeatability for measuring vitamin C intake was highest for 7dDDs (r=0.68) compared to FFQs (r=0.65) and 24-h recalls (r=0.50) [430]. Similarly, the classification of vitamin C intake into the same quartiles as those obtained with the weighed record was most consistent for the 7dDD (48%) compared to the FFQ (37%) and 24-h recall method (39%) [202, 204]. Studies have also shown that FFQs had the lowest correlations with other dietary assessment methods. For example, the between-method correlations ranged from 0.34-0.52 for FFQs and 7dDDs [201, 203, 430] and from 0.35-0.60 for FFQs and 24-hour recalls [142, 430] compared to between-method correlations of 0.63 for vitamin C intake assessed by 7dDDs and 24-hour recalls [430].

In summary, a number of validation studies have shown that 7dDDs provide more accurate estimates of dietary vitamin C intake than FFQs and 24-hour recalls.

8.2.1.2 Biological markers of vitamin C

Despite their practicality and relatively low cost, written dietary assessment methods are likely to be subjective and prone to human recall error. Moreover, they may not account for factors such as length of storage of food items, cooking practises and variations in individual bioavailability and genetic variation in metabolism of vitamin C which may influence actual intakes [207, 433]. The use of biological markers of vitamin C in plasma or serum has been suggested to overcome some of these issues [170]. For example, plasma vitamin C is thought to represent dietary vitamin C intakes from the preceding few weeks [170] and may therefore better indicate the habitual intake of the nutrient than most written dietary assessment methods. However, vitamin C in blood reflects the amount of which it is influenced by a number of biological, environmental and dietary factors. For example, increasing age has been shown to be associated with lower blood vitamin C concentrations [304], possibly due to low dietary intakes of vitamin C in older populations rather than age-related changes in vitamin C absorption and metabolism [434, 435]. Women have been shown to have higher blood levels of vitamin C than men [303, 436], which is also relative to their higher energy-adjusted dietary intake of vitamin C [224]. Moreover, blood levels of vitamin C are reduced during periods of infection [305]. Other biological factors affecting blood vitamin C levels include individual differences in vitamin C absorption into the gut, bioavailability, genetic variation in vitamin C metabolism, storage capacity, saturation levels in blood and excretion. For example, there is a renal threshold for vitamin C which results in saturated plasma concentrations at 70-85 µmol/l [300], and this corresponds to very high dietary vitamin C intakes of approximately 400 mg/d. Lifestyle factors such as smoking and body weight are also known to affect blood vitamin C levels. For example, studies have shown that smokers have lower serum vitamin C concentrations than non-smokers independent of dietary intakes of the nutrient [306, 346, 437], possibly due to an increased turnover of the nutrient [307]. Moreover, both BMI and body composition have been shown to be negatively associated with blood vitamin C concentrations [302, 304]. As humans are unable to synthesise vitamin C, the body's only source of vitamin C is through food consumption [91, 125]. Thus, circulating vitamin C concentrations are highly related to habitual intakes of foods and beverages naturally high in vitamin C such as fruit and vegetables. Studies have shown that intakes of fruit and vegetables at physiological doses are associated with plasma vitamin C concentrations [428, 429, 438]; and correlations were stronger for fruit intakes than for vegetable intakes [304, 438].

In summary, although measures of vitamin C status may overcome issues of recall bias from dietary intake assessments and inaccuracies arising from food processing, storage and preparation, blood vitamin C measures are prone to a number of dietary and biological factors which must be taken into account when interpreting this data.

8.2.1.3 The relationship between vitamin C intake and status

A number of studies have shown that the relationship between plasma vitamin C levels and quartiles or quintiles of vitamin C intake was positively correlated [429, 432, 439] **(Table 8.1)**. Correlations appear to be only modest, with ranges of 0.20-0.55 being most commonly reported [202, 303, 428-432]. The inclusion or exclusion of supplement users appears to have little effect on the relation between vitamin C intake and plasma levels [431, 432], although one study reported differently [440]. When the time point of dietary assessment was taken into account, higher within-subject correlations of up to 0.62 and higher between-subject correlations of up to 0.71 could be achieved by measuring vitamin C intake seven days and three days prior to the plasma vitamin C measurement, respectively [441]. Moreover, the type of dietary assessment has also been shown to impact on the relationship between intake and plasma levels [430, 439]. Of the different dietary assessment methods, vitamin C intake assessed by weighed records was reported to be most correlated with plasma vitamin C concentrations (r=0.49-0.51) [202]. Vitamin C intake estimated from 7-day diet diaries has been reported to be better correlated with plasma vitamin C concentrations (r=0.40-0.54) than vitamin C intake estimated from FFQs (r=0.28-0.42) and 24-hour recalls (r=0.10-0.35) [430, 431, 442], although not all studies showed consistent findings [202]. In regression analyses, linear associations between vitamin C intake and plasma levels were significantly stronger for intake assessed by a 7-day diet diary compared to a FFQ (linear trend: diary β = 5.6 mmol/l *vs.* FFQ β = 3.9 mmol/l, $P<0.05$) [439].

8.2.1.4 Vitamin C intake and status as predictors of common chronic diseases

Previous observational studies of common chronic disease outcomes or mortality have shown significant associations with both dietary intakes and plasma concentrations of vitamin C **(Table 8.2)**. Overall, the strengths of the associations were comparable between the intake and biomarker methods [439, 443]. However, in a number of studies, dietary intake of vitamin C failed to show any associations with the according chronic disease despite significant associations being found with plasma concentrations [428, 443, 444]. Thus, biomarker levels of vitamin C might be better predictors of common chronic diseases than dietary intakes estimated from questionnaires and diaries.

Table 8.1: Examples of observational studies investigating the relationship between dietary intake and biological markers of vitamin C.

Study	Subjects	Vitamin C intake	Vitamin C biomarker	Statistics	Correlation between vitamin C intake and biomarker	Further results
Bates[441] 1979 UK	*n*: 23 Men and women Age: (72-86) yrs	Daily qualitative food records for 12 months and weighed records every 6-8 weeks	Plasma and buffy-coat	Within- and between-subject correlations between intake and biomarker levels for differing periods of dietary assessment; and linear regression for sex-specific differences	Strongest within-subject correlation: r=0.62 (*P*<0.001) unadjusted (7d before plasma measurement) Strongest between-subject correlation: r=0.71 (*P*<0.001) unadjusted (3d before plasma measurement)	Duration of dietary assessment markedly affected within-subject correlations between vitamin C intake and plasma levels, whereas between-subject correlations were affected to a lesser extent. Correlations ranged from 0.22 to 0.62 within subjects (*P*<0.05) and from 0.59 to 0.71 between subjects (*P*<0.01). No sex-specific differences were found.
Jacques[432] 1993 US	*n*: 139 Men and women Age: 61 (40-83) yrs	FFQ	Plasma	Correlation between intake and plasma; and linear regression of plasma levels stratified by quartiles of intake	Data excluding supplement users: r=0.34 (*P*<0.01) unadjusted r=0.40 (*P*<0.01) energy adjusted r=0.38 (*P*<0.01) age, sex, energy adjusted Data including supplement users: r=0.39 (*P*<0.01) unadjusted r=0.44 (*P*<0.01) energy adjusted r=0.43 (*P*<0.01) age, sex, energy adjusted	There was a significant positive linear relationship between plasma vitamin C levels and quartiles of intake after adjustment for age, sex and energy intake and independent of the inclusion or exclusion of supplement users (*P*<0.001).
Bingham[440] * 1995 UK	*n*: 160 Women Age: (50-65) yrs	16d weighed record	Plasma	Pearson correlations between plasma and intake	Data excluding / including supplements: r=0.32 / r=0.86	/
Bingham[202] * 1997 UK	*n*: up to 156 Women Age: (50-65) yrs	16d weighed record, 7dDD, FFQ, 24-h recall	Plasma	Spearman correlations between plasma and intake	Data excluding supplement users: r=0.49 (weighed record) r=0.26 (FFQ) r=0.26 (recall) r=0.22 (diary) Data including supplements: r=0.51 (weighed record) r=0.35 (FFQ) r=0.34 (recall) r=0.22 (diary)	*(Note: this study studied the same sub-populations of the EPIC-Norfolk cohort as Bingham 1995.)*

Table 8.1: (continued)

Study	Subjects	Vitamin C intake	Vitamin C biomarker	Statistics	Correlation between vitamin C intake and biomarker	Further results
Bingham[430] 2001 UK	*n*: up to 237 Men and women Age: (45-75) yrs	7dDD; FFQ; 24hR (baseline and follow-up)	Plasma (baseline)	Pearson correlations between plasma and repeat intakes	r=0.40 and r=0.37 (1st and 2nd diary) r=0.28 and r=0.42 (1st and 2nd FFQ) r=0.35 and r=0.30 (1st and 2nd 24-h recall)	/
McKeown[431] 2001 UK	*n*: 134 Men and women Age: 60 yrs	7dDD; FFQ	Plasma	Spearman correlations between plasma and intake	Data excluding supplement users: r=0.52 and r=0.40 (1st and 2nd diary) r=0.44 and r=0.45 (1st and 2nd FFQ) Data including supplement users: r=0.54 and r=0.44 (1st and 2nd diary) r=0.42 and r=0.39 (1st and 2nd FFQ)	Women had higher correlation coefficients than men (e.g. diary r=0.56 *vs.* r=0.41).
Fletcher[429] 2003 UK	*n*: 1214 Men and women Age: (75-84) yrs	FFQ	Plasma	Correlation between intake and plasma; and intake stratified by quintiles of plasma levels	r=0.30 (P=*Data not shown*)	There was a significant positive linear relationship between vitamin C intake and plasma levels (*P*<0.01).
Galan[303] 2005 France	*n*: 3128 Men and women Age: 50 (35-60) yrs	Six 24hRs in 2-month intervals	Serum	Spearman partial correlation and linear regression of serum levels and intake	r=0.28 (*P*<0.0001) unadjusted r=0.31 (*P*<0.0001) age, sex, energy, BMI, alcohol, smoking adjusted	Age, vitamin C intake, energy intake, BMI and smoking explained 11% and 10% of the variance of serum vitamin C in men and women, respectively.
Wannamethee[428] 2006 UK	*n*: 3258 Men Age: 68 (60-79) yrs	FFQ (assessing past 7 days only)	Plasma	Correlation between intake and plasma	r=0.26 (*P*<0.0001)	/
Bingham[439] 2008 UK	*n*: 12474 (baseline); *n*: 7370 (follow-up); Men and women Age: (45-75) yrs	7dDD; FFQ (baseline)	Plasma (baseline and follow-up)	Linear regression of baseline and follow-up plasma levels stratified by quintiles of intake	/	Using baseline data (but not follow-up data), the diary showed significantly stronger associations with plasma levels than the FFQ (linear trend: FFQ 3.9 *vs.* diary 5.6, P<0.05; and Q1 *vs.* Q5: FFQ 16.6 *vs.* diary 23.0, P<0.05).
Vandevijvere[442] 2013 Europe	*n*: 697 Boys and girls Age: 15 yrs	24hR	Plasma	Spearman and Pearson correlation between intake and plasma	Spearman; Pearson r=0.10 (*P*=0.093); r=0.12 (*P*=0.028) (boys) r=0.17 (*P*=0.001); r=0.16 (*P*=0.003) (girls)	/

Table 8.2: Examples of observational studies assessing both dietary intake and biological markers of vitamin C for the prediction of chronic disease outcomes and mortality.

Chronic disease outcome	Study	Subjects	Vitamin C intake	Vitamin C biomarker	Statistics	Results
Cancer	Jenab[444] 2006 Europe / US	*n*: 631 Men and women Age: 59 yrs	Validated country-specific questionnaires over the past year	Plasma	ORs of gastric cancer risk stratified by quartiles of plasma and intake; OR of 1SD increase in gastric cancer risk	Higher plasma but not intake was associated with a significant reduction in gastric cancer risk (*P*=0.043). Associations with 1SD increase in risk were non-significant for both intake (OR 1.09; CI 0.90-1.33) and plasma (OR 0.93; CI 0.77-1.12), but the OR for plasma was lower than that of intake.
IHD	Wannamethee[428] 2006 UK	*n*: 3258 Men Age: 68 (60-79) yrs	FFQ (assessing past 7 days only)	Plasma	IHD markers stratified by quartiles of plasma and intake	For the majority of IHD markers measured in blood, correlations with plasma were stronger than those with intake. Moreover, plasma was associated with a number of IHD markers which were not found to be associated with intake.
IHD	Bingham[439] 2008 UK	*n*: 11134 Men and women Age: (45-75) yrs	7dDD; FFQ	Plasma	HRs of IHD risk stratified by quintiles of plasma and intake	7dDD intake predicted IHD risk to a similar extent as plasma (7dDD: linear trend HR 0.91, CI 0.86-0.96; plasma: linear trend HR 0.90, CI 0.85-0.95). In contrast, FFQ intake failed to predict IHD risk (linear trend HR 1.00, CI 0.94-1.05).
Mortality	Gale[445] 1995 UK	*n*: 730 Men and women Age: 65+ yrs	7dDD	Plasma	HRs of mortality from stroke or from coronary heart disease stratified by plasma and intake	Both intake and plasma were significantly associated with the risk of mortality from stroke across all tertiles (*P*<0.001 and *P*=0.012, respectively). Moreover, subjects with the highest compared to the lowest intake had significantly lower risk of dying from stroke (HR 0.4, 95%CI 0.2-0.6), but no such observations were found for plasma (HR 0.7, 95%CI 0.4-1.1). Although non-significant, HRs of mortality from coronary heart disease between extreme tertiles of intake and plasma were very similar (intake: HR 0.8, 95%CI 0.6-1.2; plasma: HR 0.9, 95%CI 0.6-1.3).
Mortality	Sahyoun[443] 1996 US	*n*: 725 Men and women Age: 60+ yrs	3dDD	Plasma	RRs of mortality stratified by plasma and intake	Plasma but not intake was significantly associated with the risk of mortality across all groups (*P*=0.02 and *P*=0.2, respectively). For both intake and plasma, the RR of mortality differed significantly between extreme quintiles to a similar extent (intake: HR 0.55, 95%CI 0.34-0.88; plasma: HR 0.56, 95%CI 0.34-0.91).

Abbreviations: IHD = ischemic heart disease.

8.2.2 Bone health as an outcome variable

Osteoporosis is defined as a T-score at the hip or spine of 2.5 standard deviations below the sex-specific young adult mean [59], with fractures being the clinical consequences of osteoporosis. The established standard measurement of bone density for the diagnosis of osteoporosis is DXA which determines the average amount of bone mineral of the scanned area in a two dimensional format (in g/cm^2) [60]. However, DXA only informs about the area density [63]. Although radiation exposure during a routine clinical DXA examination is very low [61], non-radiative alternative methods, such as ultrasound, may be a more favourable technique for determining bone density. Ultrasound measurements, typically performed at the heel, determine the parameters BUA (in dB/MHz) and VOS (in m/s). In contrast to DXA, BUA measures the structural organisation of bone and VOS is a measure of bone stiffness [63]. Although ultrasound is a less precise method for determining bone density than DXA and is currently not used for the diagnosis of osteoporosis, it is faster, cheaper and more portable than DXA [62]. Moreover, ultrasound has previously been shown to be capable of distinguishing bone densities of subjects with and without osteoporosis [67], and several studies have indicated that ultrasound measurements predict the risk of fractures as well as DXA measurements [68-71].

8.2.2.1 Vitamin C intake and status as predictors of bone health

To date, there is only limited evidence regarding the predictability of vitamin C intake compared with biomarkers for osteoporosis and fracture risk **(Table 8.3)**. Although studies have found associations for both dietary intakes and biomarker levels of vitamin C, the findings are contradictory. For example, studies have shown significant associations between intake and BMD loss or self-reported fractures, but no observations were found for plasma concentrations [166, 169]. As previously discussed, biomarkers of vitamin C may be better predictors of common chronic diseases than intake estimated from dietary assessments. However, whether this may also apply to osteoporosis and fracture risk is not known, as studies comparing their predictability between intake and biomarker levels of vitamin C are currently scarce. Moreover, no previous study has combined vitamin C intake with its respective biomarker when investigating the potential role of vitamin C in bone health.

Table 8.3: Observational studies of bone density and fracture risk assessing dietary intake with or without biological markers of vitamin C.

Study	Subjects	Vitamin C intake	Vitamin C biomarker	Statistics	Results	
					Vitamin C intake	**Vitamin C biomarker**
Simon[169] 2001 US	*n* 13080 Men and women Age: (20-90) yrs	24hR	Serum	TH BMD or self-reported fractures stratified by 100 mg/d increments in vitamin C intake or by SD increments in serum levels	Associations were only significant with TH BMD in pre-menopausal women and with self-reported fractures in men. In pre-menopausal women, TH BMD was 0.01 g/cm^2 higher for every 100 mg/d increase in intake (*P*=0.002). In men, associations with fractures were non-linear, with the incidence being least common at dietary intakes of around 200 mg/d, and higher or lower intakes being associated with a higher fracture prevalence (*P*=0.01).	Associations were only significant with TH BMD in men and were non-linear. TH BMD was highest at serum concentrations between approximately 28.4-56.8 µmol/l, whereas higher or lower levels were associated with lower TH BMD (*P*<0.05).
Kaptoge[166] 2003 UK	*n* 944 Men and women Age: 72 (67-79) yrs	7dDD	Plasma	2-5 year change in TH BMD stratified by tertiles of vitamin C intake and plasma levels	Associations with change in TH BMD were only significant in women. Associations were linear (*P*-trend=0.016), and those in tertiles 2 and 3 of intake had approximately 52% (*P*=0.015) and 54% (*P*=0.010) less hip BMD loss, respectively.	No significant associations between plasma levels and change in TH BMD in either sex.
Farrell[142] 2009 US	*n*: 244 Women Age: 56 yrs	8 repeat 24hRs; FFQ	/	Multiple linear regression of each intake method and BMD at five sites	There were multiple associations between intake and BMD. However, an agreement between the two dietary methods was only found at one of the five bone sites (spine BMD, FFQ β 0.170, 24-h recall β 0.155, *P*≤0.05).	/

Abbreviations: TH BMD = total hip bone mineral densit

8.2.3 Chapter aims and objectives

Dietary assessment methods are associated with measurement errors; although biomarkers of dietary intake may be better predictors of common chronic diseases by overcoming some of these issues. Moreover, combining different exposure measures may be superior to using single measures. However, to the best of my knowledge, only a limited number of studies have used this approach, and none exists on vitamin C and bone health. Therefore, the aims of this chapter were to i) evaluate the comparability of vitamin C intake estimated from different dietary assessment methods with vitamin C status, and ii) investigate whether combining intake measures may be superior to using single measures when predicting diet-bone relationships at the example of vitamin C and bone health in the EPIC-Norfolk cohort of older men and women.

The objectives were to:

i) Determine potential cross-sectional associations between i) vitamin C intake estimated from a 7dDD, ii) vitamin C intake estimated from a FFQ and iii) plasma vitamin C concentrations with measurements of heel ultrasound and DXA.

ii) Compare the predictability of fracture risk in a prospective cohort study by i) vitamin C intake estimated from a 7dDD, ii) vitamin C intake estimated from a FFQ and iii) plasma vitamin C concentrations.

iii) Assess whether combining the different dietary exposure methods into i) 7dDD+FFQ, ii) 7dDD+plasma, iii) FFQ+plasma and iv) 7dDD+FFQ+plasma may strengthen any potential associations with bone density and the predictability of fracture risk in comparison to using any of the three dietary assessment methods alone.

8.3 Methods

The following analyses were performed on a representative sample of men and women of the EPIC-Norfolk cohort study. Vitamin C was chosen as the exposure variable of interest due to i) a number of suggested underlying mechanisms in bone health, ii) modest correlations between dietary intake and blood concentrations of vitamin C and iii) the availability of intake and biomarker data in the EPIC-Norfolk study. It is well established that vitamin C is crucial to bone collagen synthesis [7-10], and recently a number of animal and cell studies suggested a role in osteoclastogenesis and osteoblastogenesis [161-164]. Moreover, the correlation between dietary or supplemental intake of vitamin C and circulating levels in blood are modest, with ranges of 0.20-0.55 being most commonly reported [202, 303, 428-432]. Furthermore, in the EPIC-Norfolk study, a range of vitamin C-related data had previously been measured. Dietary intakes of vitamin C were estimated by means of a 7dDD and a FFQ in all participants and plasma vitamin C concentrations were measured in a large number of subjects. Bone health measures included ultrasound of the heel bone which had been measured as part of the second health check and DXA measurements of the hip which had been undertaken in a small sub-sample of the cohort. Data regarding the occurrence of fractures was available from baseline up to March 2009. Details on the data collection methods of each variable can be found in Chapter 2 (page 40).

All analyses were stratified by sex. Of the random sub-cohort of 4000 participants, subjects were excluded from the subsequent analyses if they had missing data for either dietary assessment method or for plasma vitamin C concentrations. Firstly, the assessment of vitamin C intake from foods was compared between the 7dDD and the FFQ. For this, the mean (SD) vitamin C intake was calculated for each method, as the data were normally distributed, and differences in mean estimates were determined using a paired *t*-test. To assess the correlation between 7dDD and FFQ estimates, Pearson correlation coefficients were determined. Next, participants were grouped into quintiles according to their mean vitamin C intake as estimated from either method. Then, the level of agreement or misclassification between the two dietary methods was evaluated by determining the percentage of participants classified into the same or opposite quintiles, respectively.

Next, the relationship between vitamin C intakes estimated from the two dietary assessment methods with plasma vitamin C concentrations was investigated. For this, the mean (SD) plasma concentration of vitamin C was calculated. Correlations between the two intake assessment methods with plasma levels were compared using Pearson correlation coefficients. Next, the level of agreement and misclassification into the same or opposite quintiles, respectively, were compared between the two dietary intake methods with plasma concentrations of vitamin C as discussed above. We also assessed the ranking ability of the two intake methods by stratifying mean plasma vitamin C concentrations by quintiles of the 7dDD

and the FFQ using linear regression analyses. A test for trend across the quintiles of each method was indicative of the linearity of the data.

For the cross-sectional investigations of heel ultrasound in the random sub-cohort (*n*=4000), further participants were excluded from the analyses if they had missing data for the heel ultrasound measures or any of the covariates used in the multivariate model, as discussed in Chapter 3 (pages 55-57). Prior to regression analyses, vitamin C intake and status measures were divided into different quantiles separately for men and women. The distribution of the data was most suitable when using quartiles, possibly due to the smaller sample size, and hence these were used throughout the heel ultrasound analyses in this chapter. In order to compare the regression coefficients of vitamin C intake and status with heel ultrasound, standardised measures of BUA and VOS were calculated by dividing the bone measures by their standard deviation. Firstly, correlation coefficients were determined between vitamin C measures and standardised heel ultrasound measures. Then, adjusted linear regression analyses were performed for quartiles of vitamin C intake from 7dDD and from FFQ and plasma vitamin C concentrations with the standardised measures of BUA and VOS. We also combined intake and status measures of vitamin C (7dDD+FFQ, 7dDD+plasma, FFQ+plasma and 7dDD+FFQ+plasma) according to Howe's method [222], in order to determine if this may be a superior method of determining diet-bone associations than using only one dietary exposure method. Howe suggests different approaches for combining the data, and we chose the method which has previously been shown to be superior [205, 206]. For this, participants were grouped into the number of quantiles that was equal to the sample size (*n*=865 in men; *n*=1167 in women), separately for the 7dDD, the FFQ and for plasma vitamin C concentrations. Scores were subsequently calculated which reflected the quantile ranking of the participants for each method combination. The calculated scores were then re-grouped into quartiles and those were used as the explanatory variable in the linear regression analyses of 7dDD+FFQ, 7dDD+plasma, FFQ+plasma and 7dDD+FFQ+plasma with measures of heel ultrasound. All regression coefficients were compared for the linear trend across all quartiles and for differences between the higher quartiles with the lowest quartile. The linear regression analyses were adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, energy intake, dietary calcium intake, calcium supplements and vitamin D supplements.

The main bone density measurement used in the EPIC-Norfolk study was an ultrasound measurement at the heel bone which had been determined as part of the second health check, but DXA measurements at the hip were also performed in a small sub-sample of the cohort. As the latter are considered the gold standard in estimating bone density, we also performed the cross-sectional study of different measures of vitamin C intake and status in a small sub-sample of participants who had data for both the heel ultrasound and the DXA measurements. The bone

measures were standardised, as previously discussed. Due to the small sample size (*n*=151), the study was performed in a combined cohort of men and women and all analyses were adjusted for sex. The participants were grouped into tertiles (due to the small sample size) according to their vitamin C intake (7dDD, FFQ) or status prior to the linear regression analyses of heel ultrasound and DXA, and multivariate adjustment was applied as above. All regression coefficients were compared for the linear trend across all tertiles and for differences between the extreme tertiles. We also used Howe's method [(222)], as previously discussed, to determine if the combination of different exposure measurements may be superior in determining associations with heel ultrasound and DXA than using only one dietary exposure method.

For the prospective investigations of fracture risk in the case-cohort sample (*n*=5319), subjects were excluded from the subsequent analyses if they had missing data for the 7dDD, the FFQ, plasma vitamin C concentrations or any of the covariates used in the multivariate model, as discussed below. Moreover, they were also excluded if they had suffered a fracture which did not occur at the hip, spine or wrist and they were not part of the random sub-cohort. In the present investigations, the combined sum of fractures at the hip, spine and wrist (total fractures) was used to increase the power of the study to detect potential prospective associations between vitamin C and fracture risk. Similar to the heel ultrasound investigations, intake and plasma measures of the vitamin C were divided into sex-specific quartiles. Then, Prentice-weighted Cox proportional hazard ratios were calculated for quartiles of vitamin C intake from 7dDD and from FFQ and plasma vitamin C concentrations with total fracture risk after the median follow-up of 12.9 years. We also combined intake and status measures of vitamin C according to Howe's method [(222)], as previously discussed, before calculating further hazard ratios for the combined measures (7dDD+FFQ, 7dDD+plasma, FFQ+plasma and 7dDD+FFQ+plasma). We compared the hazard ratios for the linear trend across all quartiles and for differences between the higher quartiles with the lowest quartile of vitamin C. The calculations of all hazard ratios were adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, energy intake, dietary calcium intake, calcium supplements and vitamin D supplements.

8.4 Results

8.4.1 The relationship between 7dDD and FFQ estimates of vitamin C intake

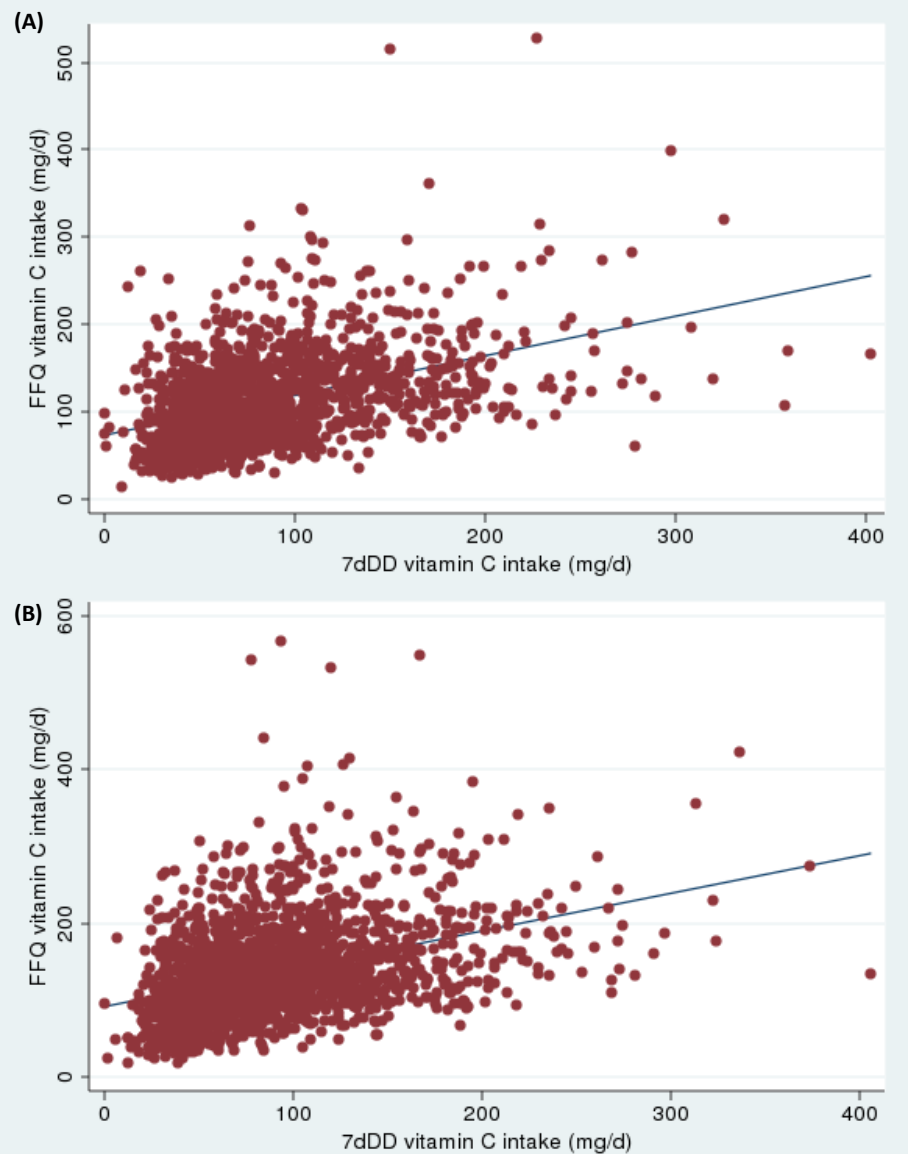
Following the exclusion of those with missing data (*n*=598) in the random sub-cohort sample as discussed previously, 3402 participants (55% women) with a mean age of 60±10 years remained for analysis. Firstly, the assessment of vitamin C intake from foods was compared between the two different dietary assessment methods 7dDD and FFQ **(Table 8.4)**. Mean±SD dietary vitamin C intakes estimated from the 7dDD were 86.7±51.2 mg/d and 90.3±49.6 mg/d for men and women, respectively, whereas mean intakes from the FFQ were 30% and 50% higher (all *P*<0.001). Pearson correlation coefficients indicated that vitamin C intake estimated from the 7dDD was moderately correlated with that of the FFQ, with significant correlation coefficients of 0.44 in men and 0.39 in women (*P*<0.05) **(Figure 8.1)**.

Table 8.4: Estimates of dietary vitamin C intake from 7dDD and FFQ.

	Vitamin C intake (mg/d) estimated from				
	7dDD		FFQ		
	Mean	(SD)	Mean	(SD)	*P*
Men	86.7	(51.2)	113.0	(52.8)	*P*<0.001
Women	90.3	(49.6)	135.3	(62.9)	*P*<0.001

n=1520 men and n=1882 women.

Figure 8.1: The correlation of vitamin C intake estimated from 7dDD and FFQ**.**



*The Pearson correlation coefficients were **(A)** r=0.44 (P<0.05) in men and **(B)** r=0.39 (P<0.05) in women. n=1520 men and 1882 women.*

Next, the extent to which the two dietary assessment methods were able to classify individuals into the same quintile of vitamin C intake was investigated **(Table 8.5)**. The amount of misclassification into the extreme quintiles was also determined. In both sexes, the highest level of agreement was found for the lowest quintile of vitamin C intake, with 43-45% of participants being classified into this quintile using both the 7dDD and the FFQ. The top quintile of vitamin C intake showed the second highest agreement with 39% in men and 35% in women. The agreement between quintiles was much lower in quintiles 2-4, with the percentage agreement ranging from 23-25% in men and 20-25% in women. In both sexes, 2-8% of participants were misclassified into the opposite quintiles of vitamin C intake.

Table 8.5: The agreement and disagreement between 7dDD and FFQ.

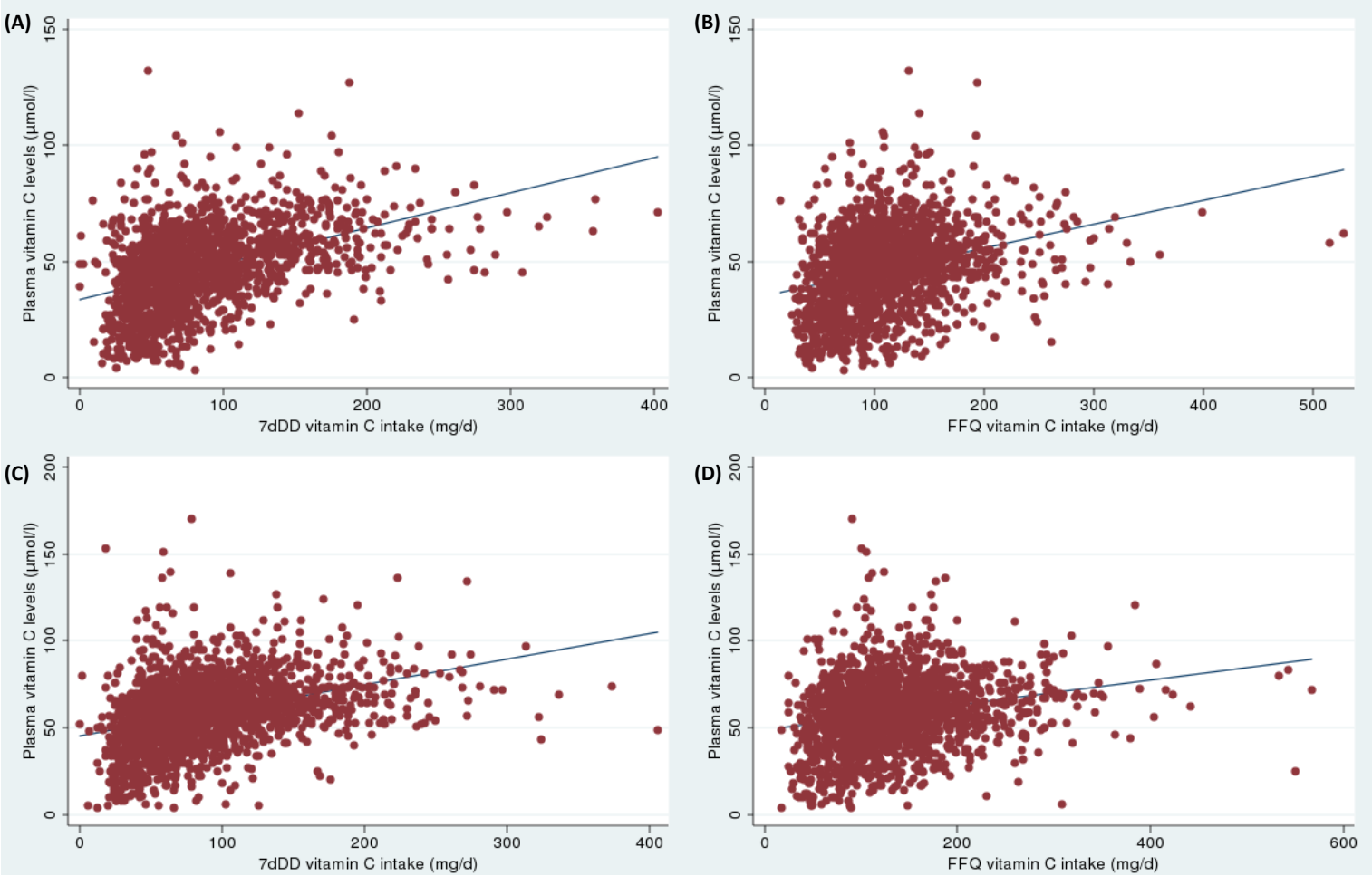
	Quintiles of vitamin C intake	**Classification into quintiles (%)**	
	7dDD *vs.* FFQ	**Men**	**Women**
Agreement	Q1 *vs.* Q1	43	45
	Q2 *vs.* Q2	23	22
	Q3 *vs.* Q3	25	20
	Q4 *vs.* Q4	24	25
	Q5 *vs.* Q5	39	35
Disagreement	Q1 *vs.* Q5	8	8
	Q5 *vs.* Q1	2	3

Values are the percentage classification of participants into the same quintiles (agreement) and extreme quintiles (disagreement) of 7dDD and FFQ estimates of vitamin C intake. n=1520 men and n=1882 women.

8.4.2 The relationship between 7dDD and FFQ estimates of dietary intake with blood levels of vitamin C

Mean plasma vitamin C concentrations were 46.9±18.0 µmol/l in men and 58.6±20.0 µmol/l in women. Firstly, the correlation coefficients of vitamin C intake and plasma concentrations were determined. All correlations were significant (all $P<0.05$), although correlations in men appeared to be higher than those in women **(Figure 8.2)**. Vitamin C intake estimated from the 7dDD was better correlated with plasma vitamin C concentrations than the FFQ in both men (r=0.44 *vs.* r=0.30) and women (r=0.37 *vs.* r=0.22).

Figure 8.2: Correlations of 7dDD and FFQ estimates of vitamin C intake with plasma status.



*The Pearson correlation coefficients were **(A)** r=0.44 (P<0.05) between 7dDD and plasma vitamin C in men, **(B)** r=0.30 (P<0.05) between FFQ and plasma vitamin C in men, **(C)** r=0.37 (P<0.05) between 7dDD and plasma vitamin C in women and **(D)** r=0.22 (P<0.05) between FFQ and plasma vitamin C in women. n=1520 men and n=1882 women.*

Next, the relationship between vitamin C intakes assessed by either dietary assessment method compared to plasma vitamin C concentrations was further investigated by determining the level of agreement of classifying participants into the same quintile **(Table 8.6)**. The agreement between quintiles of intake and plasma levels ranged from 22-45% for the 7dDD and from 22-44% for the FFQ. The classifications were comparable between the 7dDD and the FFQ for all quintiles except for quintile 5. The latter showed much higher agreement with quintiles of plasma vitamin C for the 7dDD (41% in men, 34% in women) compared to the FFQ (28% in men, 27% in women). A similar discrepancy was found in women of quintile 1, where the agreement was 45% for the 7dDD but only 38% for the FFQ.

Table 8.6: The agreement between 7dDD and FFQ with plasma vitamin C.

	Agreement between quintiles of vitamin C (%)			
	Men		**Women**	
Quintile	**7dDD *vs.* plasma**	**FFQ *vs.* plasma**	**7dDD *vs.* plasma**	**FFQ *vs.* plasma**
Q1	45	44	45	38
Q2	25	25	24	22
Q3	22	22	23	23
Q4	22	26	27	22
Q5	41	28	34	27

n=1520 men and n=1882 women.

Then, plasma vitamin C concentrations were classified into quintiles of dietary vitamin C intake estimated from i) the 7dDD and ii) the FFQ **(Table 8.7)**. Plasma vitamin C concentrations increased significantly across quintiles of intake for both dietary assessment methods in a linear fashion in both sexes (all $P<0.001$). In both men and women, mean plasma levels increased at relatively consistent intervals when stratified by 7dDD intake estimates. In contrast, the classification by FFQ was less consistent. Mean plasma concentrations increased from quintile 1 to quintile 2 by an interval of 9.7 µmol/l in men and 8.5 µmol/l in women. This was followed by much smaller intervals of 1.1-4.0 µmol/l in men and 0.8-2.7 µmol/l in women for the remaining quintiles.

Table 8.7: Changes in plasma vitamin C across quintiles of 7dDD and FFQ vitamin C intake.

			Plasma vitamin C concentrations (µmol/l) by quintiles of dietary vitamin C intake					
			7dDD			**FFQ**		
	Quintile	***n***	**Mean**	**(SD)**	**[Range]**	**Mean**	**(SD)**	**[Range]**
Men	Q1	304	35.3	(17.4)	[4 – 96]	35.9	(17.5)	[4 – 95]
	Q2	304	41.8	(17.4)	[6 – 132]	45.4	(16.9)	[3 – 101]
	Q3	304	46.3	(16.5)	[3 – 104]	47.9	(17.8)	[6 – 106]
	Q4	304	51.3	(14.4)	[12 – 106]	52.0	(17.2)	[9 – 132]
	Q5	304	59.8	(14.1)	[23 – 127]	53.4	(15.3)	[14 – 127]
					P-trend<0.001			*P*-trend<0.001
Women	Q1	377	45.7	(20.9)	[4 – 153]	49.0	(21.2)	[4 – 116]
	Q2	376	55.5	(20.4)	[4 – 151]	59.0	(21.5)	[4 – 170]
	Q3	377	59.0	(16.9)	[8 – 170]	59.2	(17.8)	[14 – 140]
	Q4	376	63.7	(16.6)	[6 – 139]	61.7	(18.7)	[6 – 127]
	Q5	376	69.2	(16.6)	[6 – 136]	64.1	(17.2)	[6 – 136]
					P-trend<0.001			*P*-trend<0.001

n=1520 men and n=1882 women.

8.4.3 Cross-sectional associations between vitamin C estimated from different exposure assessments and heel ultrasound

Following the exclusion of participants with further missing data of heel ultrasound measurements and covariate information, 865 men and 1167 women remained for the following cross-sectional analyses of vitamin C and heel ultrasound. Firstly, BUA and VOS were standardised for comparison reasons using their respective standard deviations (sBUA, sVOS), and the results of these calculations are shown in **Table 8.8**. Standardised BUA was 5.2 and 4.4 dB/MHz/SD in men and women respectively, and sVOS was 41.3 and 40.4 m/s/SD respectively.

Table 8.8: Mean BUA and VOS as crude and standardised values.

	BUA (dB/MHz)		sBUA (dB/MHz/SD)		VOS (m/s)		sVOS (m/s/SD)	
	Mean	**(SD)**	**Mean**	**(SD)**	**Mean**	**(SD)**	**Mean**	**(SD)**
Men	90	(17)	5.2	(1)	1645	(40)	41.3	(1)
Women	72	(16)	4.4	(1)	1624	(40)	40.4	(1)

Abbreviations: sBUA and sVOS, standardised BUA and VOS. n=865 men and n=1167 women.

Pearson correlation coefficients between standardised heel ultrasound measurements and vitamin C were very small and predominantly non-significant **(Table 8.9)**. Only vitamin C intake estimated from the 7dDD correlated significantly positively with both sBUA (r=0.07) and sVOS (r=0.06, both *P*<0.05) in women only.

Table 8.9: Correlations between BUA and VOS with vitamin C estimates from 7dDD, FFQ and plasma.

	Men		Women	
	sBUA	sVOS	sBUA	sVOS
7dDD vitamin C intake	-0.01	0.04	0.07*	0.06*
FFQ vitamin C intake	-0.04	-0.03	-0.03	-0.02
Plasma vitamin C levels	0.00	0.05	0.04	0.04

Abbreviations: sBUA and sVOS, standardised BUA and VOS.
n=865 men and n=1167 women.
**Pearson correlation coefficients were significant at P<0.05.*

Due to the smaller sample size in the bone health investigations, participants were classified into quartiles rather than quintiles of vitamin C intake and status for the following analyses. Results from the linear regression analyses of the different vitamin C exposure measurements and measures of heel ultrasound are shown in **Table 8.10** for men and in **Table 8.11** for women. All β-coefficients are shown adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT use in women, energy intake, dietary calcium intake, calcium supplements and vitamin D supplements.

In men, associations between quartiles of vitamin C intake (7dDD, FFQ) and status (plasma) with sBUA and sVOS were non-significant **(Table 8.10)**. Despite the lack of statistical

significance, the β-coefficients of the 7dDD investigating the linearity of the data and potential differences between quartiles were all positive in contrast to those of the FFQ which were predominantly negative. The β-coefficients of plasma vitamin C were of mixed nature, although coefficients were predominantly positive. We also combined the different measures of vitamin C in order to investigate whether their combination may be superior to using them separately. When measures were combined in men, the associations with heel ultrasound remained non-significant, except for a significant difference in sVOS for men in quartile 2 compared to quartile 1 of 7dDD+plasma (β 0.206±0.096 m/s, *P*=0.031). However, the combination of 7dDD+plasma compared to using separate measures improved most β-coefficients. For example, coefficients for quartile 4 *vs.* 1 were 0.164±0.099 m/s (*P*=0.10) for the 7dDD intake and 0.024±0.101 m/s (*P*=0.81) for plasma concentrations, but 0.177±0.100 m/s (*P*=0.08) for 7dDD+plasma. Both the 7dDD+FFQ measure and the combination of all three vitamin C estimates (7dDD+FFQ+plasma) mainly strengthened the associations between vitamin C and heel ultrasound in a similar fashion. In contrast, the combined FFQ+plasma measure showed only minor improvements as the associations remained predominantly negative but non-significant.

In women, there was a significant linear trend across quartiles of intake estimated from the 7dDD (β 0.063±0.023 dB/MHz per quartile, *P*-trend=0.007), and women in quartile 3 (β 0.144±0.072 dB/MHz, *P*=0.047) and quartile 4 (β 0.180±0.074 dB/MHz, *P*=0.015) of 7dDD intake had significantly higher sBUA compared to women in quartile 1 **(Table 8.11)**. Moreover, although non-significant, there was a trend for a linear relationship between sVOS and 7dDD intake (β 0.044±0.024 m/s per quartile, *P*-trend=0.07) and a trend for an association between sVOS and extreme quartiles of 7dDD intake (β 0.137±0.075 m/s, *P*=0.07). In contrast, intake estimated from the FFQ was not associated with either sBUA or sVOS. For plasma vitamin C concentrations, a significant difference in women of quartile 2 compared to quartile 1 of was found for both sBUA (β 0.153±0.070 dB/MHz, *P*=0.030) and sVOS (β 0.174±0.072 m/s, *P*=0.016), but no associations were found for the upper quartiles. When the different vitamin C measurements were combined, associations with 7dDD+FFQ, FFQ+plasma and 7dDD+FFQ+plasma were non-significant. However, women in quartile 2 (β 0.154±0.071 dB/MHz, *P*=0.031) and quartile 4 (β 0.185±0.074 dB/MHz, *P*=0.012) of 7dDD+plasma compared to those women in quartile 1 had significantly higher sBUA, and the test for linearity across all quartiles was almost significant (β 0.045±0.023 dB/MHz per quartile, *P*-trend=0.052). Interestingly, in contrast to men, the combination of vitamin C measures did not necessarily improve the strength of the associations.

8.4.3.1 Cross-sectional associations with heel ultrasound and DXA

As DXA measurements are considered the gold standard method for determining bone density, and DXA measurements at the hip were available in a small sub-sample of EPIC-Norfolk participants, the cross-sectional study of dietary intake and plasma concentration estimates of vitamin C was also performed in a small sub-sample of 151 participants (52% men) who had data for both the heel ultrasound measurements and the DXA measurements. All analyses were adjusted for age, sex, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT use in women, energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. In this study, the results from the linear regression analyses showed that there were no significant associations between any of the vitamin C measures (single or combined) and either standardised measurement of bone density (all $P \geq 0.05$, *Data not shown*). For example, associations between tertiles of vitamin C and the standardised DXA measurements were (β 0.132±0.085 g/cm^2/SD per tertile, $P=0.12$) for the 7dDD vitamin C intake, (β 0.038±0.084 g/cm^2/SD per tertile, $P=0.65$) for the FFQ vitamin C intake and (β 0.077±0.087 g/cm^2/SD per tertile, $P=0.38$) for plasma vitamin C.

Table 8.10: Associations between quartiles of vitamin C (7dDD, FFQ, plasma and their respective combinations) with BUA and VOS in 865 men.

		Q2 *vs.* Q1	**Q3 *vs.* Q1**	**Q4 *vs.* Q1**	**Linear trend across all Qs**
sBUA	7dDD	β 0.007 ± 0.097; *P*=0.94	β 0.026 ± 0.100; *P*=0.80	β 0.013 ± 0.100; *P*=0.89	β 0.006 ± 0.032; *P*=0.86
	FFQ	β -0.118 ± 0.096; *P*=0.22	β 0.019 ± 0.096; *P*=0.84	β -0.096 ± 0.098; *P*=0.33	β -0.015 ± 0.031; *P*=0.63
	Plasma	β 0.005 ± 0.095; *P*=0.96	β 0.040 ± 0.097; *P*=0.68	β -0.045 ± 0.102; *P*=0.66	β -0.009 ± 0.032; *P*=0.78
	7dDD+FFQ	β -0.152 ± 0.096; *P*=0.11	β 0.034 ± 0.097; *P*=0.72	β -0.069 ± 0.099; *P*=0.49	β -0.002 ± 0.031; *P*=0.96
	7dDD+Plasma	β 0.093 ± 0.097; *P*=0.34	β 0.049 ± 0.099; *P*=0.62	β 0.035 ± 0.101; *P*=0.73	β 0.006 ± 0.032; *P*=0.86
	FFQ+Plasma	β -0.004 ± 0.096; *P*=0.96	β -0.012 ± 0.098; *P*=0.90	β -0.091 ± 0.099; *P*=0.36	β -0.028 ± 0.031; *P*=0.37
	7dDD+FFQ+Plasma	β -0.005 ± 0.096; *P*=0.96	β 0.059 ± 0.097; *P*=0.55	β -0.053 ± 0.100; *P*=0.60	β -0.009 ± 0.032; *P*=0.77
sVOS	7dDD	β 0.038 ± 0.096; *P*=0.69	β 0.133 ± 0.099; *P*=0.18	β 0.164 ± 0.099; *P*=0.10	β 0.059 ± 0.032; *P*=0.06
	FFQ	β -0.045 ± 0.095; *P*=0.63	β 0.039 ± 0.095; *P*=0.69	β -0.046 ± 0.097; *P*=0.64	β -0.005 ± 0.031; *P*=0.86
	Plasma	β 0.074 ± 0.094; *P*=0.43	β 0.113 ± 0.096; *P*=0.24	β 0.024 ± 0.101; *P*=0.81	β 0.012 ± 0.032; *P*=0.70
	7dDD+FFQ	β -0.140 ± 0.095; *P*=0.14	β 0.158 ± 0.095; *P*=0.10	β -0.014 ± 0.098; *P*=0.89	β 0.026 ± 0.031; *P*=0.40
	7dDD+Plasma	**β 0.206 ± 0.096; *P*=0.031**	β 0.180 ± 0.097; *P*=0.07	β 0.177 ± 0.100; *P*=0.08	β 0.050 ± 0.032; *P*=0.12
	FFQ+Plasma	β 0.084 ± 0.095; *P*=0.38	β 0.082 ± 0.097; *P*=0.40	β -0.036 ± 0.098; *P*=0.71	β -0.012 ± 0.031; *P*=0.71
	7dDD+FFQ+Plasma	β 0.020 ± 0.095; *P*=0.84	β 0.121 ± 0.096; *P*=0.21	β 0.017 ± 0.099; *P*=0.86	β 0.016 ± 0.031; *P*=0.62

Abbreviations: sBUA and sVOS, standardised BUA and VOS. Values are adjusted β-coefficients ± SE. Measures of vitamin C were combined using Howe's method of ranks [(222)].

Table 8.11: Associations between quartiles of vitamin C (7dDD, FFQ, plasma and their respective combinations) with BUA and VOS in 1167 women.

		Q2 *vs.* Q1	Q3 *vs.* Q1	Q4 *vs.* Q1	Linear trend across all Qs
sBUA	7dDD	β 0.053 ± 0.072; *P*=0.46	**β 0.144 ± 0.072; *P*=0.047**	**β 0.180 ± 0.074; *P*=0.015**	**β 0.063 ± 0.023; *P*=0.007**
	FFQ	β 0.037 ± 0.071; *P*=0.61	β -0.048 ± 0.072; *P*=0.50	β -0.040 ± 0.072; *P*=0.58	β -0.020 ± 0.023; *P*=0.38
	Plasma	**β 0.153 ± 0.070; *P*=0.030**	β 0.083 ± 0.073; *P*=0.26	β 0.097 ± 0.074; *P*=0.19	β 0.022 ± 0.023; *P*=0.35
	7dDD+FFQ	β 0.022 ± 0.071; *P*=0.75	β 0.007 ± 0.072; *P*=0.92	β 0.083 ± 0.073; *P*=0.26	β 0.023 ± 0.023; *P*=0.31
	7dDD+Plasma	**β 0.154 ± 0.071; *P*=0.031**	β 0.054 ± 0.072; *P*=0.45	**β 0.185 ± 0.074; *P*=0.012**	β 0.045 ± 0.023; *P*=0.05
	FFQ+Plasma	β 0.127 ± 0.072; *P*=0.08	β -0.023 ± 0.072; *P*=0.75	β -0.009 ± 0.073; *P*=0.90	β -0.014 ± 0.023; *P*=0.55
	7dDD+FFQ+Plasma	β 0.063 ± 0.072; *P*=0.38	β 0.031 ± 0.072; *P*=0.67	β 0.063 ± 0.074; *P*=0.39	β 0.016 ± 0.023; *P*=0.50
sVOS	7dDD	β 0.015 ± 0.073; *P*=0.84	β 0.041 ± 0.074; *P*=0.58	β 0.137 ± 0.075; *P*=0.07	β 0.044 ± 0.024; *P*=0.07
	FFQ	β 0.100 ± 0.073; *P*=0.17	β 0.024 ± 0.074; *P*=0.75	β 0.073 ± 0.074; *P*=0.32	β 0.015 ± 0.023; *P*=0.53
	Plasma	**β 0.174 ± 0.072; *P*=0.016**	β 0.102 ± 0.075; *P*=0.17	β 0.047 ± 0.075; *P*=0.54	β 0.006 ± 0.024; *P*=0.79
	7dDD+FFQ	β -0.030 ± 0.073; *P*=0.68	β -0.016 ± 0.074; *P*=0.82	β 0.047 ± 0.075; *P*=0.53	β 0.015 ± 0.024; *P*=0.51
	7dDD+Plasma	β 0.065 ± 0.073; *P*=0.38	β 0.049 ± 0.074; *P*=0.51	β 0.108 ± 0.075; *P*=0.15	β 0.031 ± 0.024; *P*=0.20
	FFQ+Plasma	β 0.113 ± 0.074; *P*=0.12	β 0.071 ± 0.074; *P*=0.34	β 0.010 ± 0.075; *P*=0.90	β -0.002 ± 0.024; *P*=0.94
	7dDD+FFQ+Plasma	β 0.055 ± 0.073; *P*=0.45	β 0.067 ± 0.074; *P*=0.37	β 0.083 ± 0.075; *P*=0.27	β 0.026 ± 0.024; *P*=0.27

Abbreviations: sBUA and sVOS, standardised BUA and VOS. Values are adjusted β-coefficients ± SE. Measures of vitamin C were combined using Howe's method of ranks [(222)].

8.4.4 Prospective associations between vitamin C estimated from different exposure assessments and total fracture risk

In order to increase the power to detect potential prospective associations between vitamin C and fracture risk, the following investigations were based on total fractures, describing the combined sum of fractures at the hip, spine and wrist. Moreover, participants were classified into quartiles rather than quintiles of vitamin C intake and status. Following the exclusion of participants with missing data and of those not part of the random sub-cohort who had a fracture which had not occurred at the hip, spine or wrist, 1702 men and 2308 women remained for analysis in this case-cohort sample. The results from the calculations of Prentice-weighted Cox proportional hazard ratios (HRs) are shown in **Table 8.12**. All HRs were adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, steroid medication, menopausal status and HRT in women, energy intake, dietary calcium intake and calcium and vitamin D supplements.

In men, there were significant associations between total fracture risk and FFQ intake as well as plasma concentrations, but not between fracture risk and 7dDD intake. For example, men in quartile 3 compared to those in quartile 1 of FFQ intake (HR 0.55, 95%CI 0.36-0.85; *P*=0.006) and of plasma concentrations (HR 0.54, 95%CI 0.35-0.84; *P*=0.006) had a significantly lower risk of total fracture. Moreover, the trend across all quartiles was also significant and comparable between the two methods of vitamin C assessment (FFQ: HR 0.86, 95%CI 0.75-0.99; *P*=0.040; plasma: HR 0.86, 95%CI 0.74-0.99; *P*=0.041). When the different exposure measures were combined, the HR indicating the linearity of the association improved slightly for FFQ+plasma (HR 0.83, 95%CI 0.73-0.96; *P*=0.010), and the associations between the different quartiles of FFQ+plasma were now significant between extreme quartiles rather than quartile 3 (HR 0.55, 95%CI 0.35-0.86; *P*=0.009). In contrast, combining only the two intake measures (7dDD+FFQ) lead to non-significant associations between vitamin C and total fracture risk. Interestingly, the combined 7dDD+plasma measure improved the associations compared to those of the assessment methods alone. For example, the association between extreme quartiles of vitamin C and total fracture risk were not significant for the 7dDD intake (HR 0.73, 95%CI 0.48-1.13; *P*=0.16) and plasma concentrations (HR 0.71, 95%CI 0.46-1.09; *P*=0.12) when used alone, but combining the measures to 7dDD+plasma strengthened the association and it gained significance (HR 0.55, 95%CI 0.35-0.86; *P*=0.010). Slight improvements were also found for the trend across quartiles of 7dDD+plasma (HR 0.83, 95%CI 0.72-0.96; *P*=0.012). The 7dDD+FFQ+plasma measure showed some improvements in the strength of the association compared to the use of separate measures, particularly for the difference between extreme quartiles of vitamin C. However, this measure showed less improvement compared to the 7dDD+plasma and the FFQ+plasma measures.

In women, vitamin C was not associated with total fracture risk, independent of the assessment method used. Interestingly, the calculated HRs were comparable between the 7dDD intake and plasma concentrations but less so with FFQ intake, in contrast to men where HRs were comparable between the FFQ and plasma levels. For example, in women, the non-significant HRs for differences between extreme quartiles were 1.20 (95%CI 0.86-1.66, *P*=0.28) for 7dDD intake, 1.21 (95%CI 0.88-1.67, *P*=0.23) for plasma concentrations but 1.07 (95%CI 0.79-1.44, *P*=0.67) for FFQ intake. In contrast to men, combining the different measures of vitamin C did not improve the results in women.

Table 8.12: Total fracture risk stratified by quartiles of vitamin C (7dDD, FFQ, plasma and their respective combinations).

	Q2 *vs.* Q1	**Q3 *vs.* Q1**	**Q4 *vs.* Q1**	**Linear trend across all Qs**
Men				
7dDD	HR 0.67, 95%CI 0.44-1.03; *P*=0.07	HR 0.73, 95%CI 0.48-1.13; *P*=0.16	HR 0.73, 95%CI 0.48-1.13; *P*=0.16	HR 0.91, 95%CI 0.79-1.06; *P*=0.22
FFQ	HR 0.80, 95%CI 0.53-1.21; *P*=0.30	**HR 0.55, 95%CI 0.36-0.85; *P*=0.006**	HR 0.70, 95%CI 0.46-1.07; *P*=0.10	**HR 0.86, 95%CI 0.75-0.99; *P*=0.040**
Plasma	HR 0.80, 95%CI 0.54-1.19; *P*=0.27	**HR 0.54, 95%CI 0.35-0.84; *P*=0.006**	HR 0.71, 95%CI 0.46-1.09; *P*=0.12	**HR 0.86, 95%CI 0.74-0.99; *P*=0.041**
7dDD+FFQ	HR 0.77, 95%CI 0.51-1.17; *P*=0.22	HR 0.77, 95%CI 0.51-1.16; *P*=0.22	HR 0.70, 95%CI 0.45-1.09; *P*=0.12	HR 0.90, 95%CI 0.78-1.03; *P*=0.13
7dDD+Plasma	HR 0.76, 95%CI 0.50-1.14; *P*=0.19	HR 0.73, 95%CI 0.48-1.11; *P*=0.14	**HR 0.55, 95%CI 0.35-0.86; *P*=0.010**	**HR 0.83, 95%CI 0.72-0.96; *P*=0.012**
FFQ+Plasma	HR 0.83, 95%CI 0.56-1.23; *P*=0.34	HR 0.77, 95%CI 0.51-1.17; *P*=0.23	**HR 0.55, 95%CI 0.35-0.86; *P*=0.009**	**HR 0.83, 95%CI 0.73-0.96; *P*=0.010**
7dDD+FFQ+Plasma	HR 0.76, 95%CI 0.50-1.15; *P*=0.19	HR 0.94, 95%CI 0.63-1.41; *P*=0.76	**HR 0.62, 95%CI 0.39-0.97; *P*=0.036**	HR 0.89, 95%CI 0.77-1.02; *P*=0.09
Women				
7dDD	HR 1.16, 95%CI 0.85-1.59; *P*=0.35	HR 1.02, 95%CI 0.74-1.39; *P*=0.92	HR 1.20, 95%CI 0.86-1.66; *P*=0.28	HR 1.04, 95%CI 0.94-1.15; *P*=0.45
FFQ	HR 1.02, 95%CI 0.74-1.39; *P*=0.93	HR 0.91, 95%CI 0.66-1.24; *P*=0.53	HR 1.07, 95%CI 0.79-1.44; *P*=0.67	HR 1.01, 95%CI 0.92-1.11; *P*=0.83
Plasma	HR 1.17, 95%CI 0.86-1.58; *P*=0.32	HR 1.02, 95%CI 0.75-1.40; *P*=0.89	HR 1.21, 95%CI 0.88-1.67; *P*=0.23	HR 1.05, 95%CI 0.95-1.16; *P*=0.38
7dDD+FFQ	HR 1.10, 95%CI 0.81-1.49; *P*=0.54	HR 0.94, 95%CI 0.69-1.28; *P*=0.69	HR 1.12, 95%CI 0.82-1.54; *P*=0.47	HR 1.02, 95%CI 0.92-1.13; *P*=0.72
7dDD+Plasma	HR 1.02, 95%CI 0.75-1.38; *P*=0.92	HR 1.18, 95%CI 0.87-1.61; *P*=0.29	HR 0.99, 95%CI 0.72-1.38; *P*=0.97	HR 1.01, 95%CI 0.92-1.12; *P*=0.78
FFQ+Plasma	HR 1.29, 95%CI 0.95-1.76; *P*=0.11	HR 1.12, 95%CI 0.82-1.54; *P*=0.47	HR 1.14, 95%CI 0.83-1.56; *P*=0.43	HR 1.02, 95%CI 0.93-1.13; *P*=0.63
7dDD+FFQ+Plasma	HR 1.18, 95%CI 0.87-1.60; *P*=0.28	HR 0.98, 95%CI 0.72-1.34; *P*=0.89	HR 1.12, 95%CI 0.81-1.54; *P*=0.50	HR 1.02, 95%CI 0.92-1.12; *P*=0.77

Values are adjusted Prentice-weighted Cox proportional hazard ratios of total fracture risk. Measures of vitamin C were combined using Howe's method of ranks [(222)]. n=1702 men and n=2308 women.

8.5 Discussion

The main findings of the present study were that combining dietary intake and plasma estimates of vitamin C using different dietary assessment methods compared to single measures strengthened the associations between vitamin C with both heel ultrasound and fracture risk in men, but not in women.

Dietary assessment methods are known to inaccurately reflect estimates of habitual food intake in populations due to measurement errors associated with their use. However, combining different exposure measures may be superior to using single measures, as this approach has previously been shown to potentially increase the power of studies in detecting diet-disease associations [205]. To the best of my knowledge, only a limited number of studies have used this approach and none exist on bone health. Assuming that vitamin C is positively associated with bone health due to its crucial role in bone collagen synthesis [7-10], we aimed to investigate vitamin C estimated from three different exposure methods (dietary intake from a 7dDD and a FFQ, and plasma concentrations), and their respective combinations, and compare their ability to detect i) the cross-sectional association between vitamin C and heel ultrasound and DXA, and ii) the prospective association between vitamin C and fracture risk. Our results, showing that combining different measures strengthened the associations with heel ultrasound in men but not in women, are completely novel.

8.5.1 Heel ultrasound

In detail, in the cross-sectional study of heel ultrasound, we found that in men, associations between quartiles of vitamin C and heel ultrasound were non-significant, independent of the assessment method used. Interestingly, using single measures, the 7dDD and plasma showed positive trends, whereas the FFQ showed a negative trend. Assuming that vitamin C is associated with bone health, we aimed to investigate whether different ways of combining the exposure methods would give superior estimates of the associations compared to using single methods. We first combined the two intake measures. Their combination may improve the detection of an association between vitamin C and bone health, but only to a minor extent, possibly because their measurement errors may correlate with one another [194, 293]. In contrast, measures of intake and blood concentrations have unrelated errors, thus their combination may provide a superior way of detecting the associations between vitamin C and measures of bone health. We found that the different combinations (7dDD+FFQ, 7dDD+plasma and 7dDD+FFQ+plasma) mainly improved the associations, although only minor improvements were found when combining the FFQ with plasma. The latter finding may be due to the 7dDD having been shown to give more accurate estimates of vitamin C compared to a FFQ [201-204]. Thus, one may expect to find that the combination of the 7dDD with plasma levels may be superior to combining a FFQ

with a biomarker. In women, using separate measures, significant associations were only found for the 7dDD and sBUA as well as a trend for an association with sVOS. The combination of the 7dDD with plasma reflected those associations, but all other combinations of measures (7dDD+FFQ, FFQ+plasma and 7dDD+FFQ+plasma) showed no significant associations. However, in contrast to men, combining measures in women did not consistently lead to improved associations between vitamin C and heel ultrasound, but we are not sure why this might be.

8.5.2 DXA measurements

We also performed the cross-sectional study of different measures of vitamin C intake and status in a small sub-sample of participants who had additional data for DXA measurements at the hip, as DXA is regarded the gold standard method for measuring bone density [(446)]. In the present study, there were no significant associations between any of the vitamin C measures, neither as single measures nor as their respective combinations, and both heel ultrasound measurements and DXA. It is likely that this was due to the small sample size of only 151 participants which had too little power to detect any significant diet-bone associations.

8.5.3 Fracture risk

The prospective fracture risk investigations showed similar sex-specific findings for the superiority of combined measures as with the cross-sectional study of heel ultrasound. In men, using separate measures, total fracture risk was significantly lower with higher vitamin C intakes estimated from the FFQ and with plasma concentrations, and the HRs were comparable between these methods. There were no associations with the 7dDD. Apart from the combination of the two intake measures (7dDD+FFQ), all other combinations of measures showed significant associations with fracture risk and the HRs indicated stronger associations. Although the combination of all three measures improved the associations, the combination of an intake measure with a blood concentration measure (7dDD+plasma, FFQ+plasma) showed the strongest associations with total fracture risk. The latter findings may relate to the correlation of errors in the combined variable of exposure measures [(293)]. This is because when combining two intake measures with a biomarker, the measurement errors from the intake measures would be expected to correlate with one another, whereas those from the intake and plasma measures would not. Thus, the idea of combining two intake measures with a biomarker may not necessarily provide a better way of detecting diet-disease associations compared to using a combination of two methods, but may still be superior to using only a single exposure assessment method. In women, there were no significant associations between vitamin C and total fracture risk, independent of the assessment method used. In contrast to men, the combination of different measures in women did not improve the associations. Moreover, the

HRs were comparable between the 7dDD and plasma levels and not between the FFQ and plasma as seen in men, but we are not sure why this might be.

To the best of my knowledge, only a limited number of studies have used the approach of combining different exposure measures in order to potentially increase the statistical power of the diet-disease association [205]. For example, one prospective cohort study investigated the associations between dietary lutein plus zeaxanthin and the risk of nuclear cataracts and nuclear sclerosis [206]. The study was based on dietary intakes estimated from a FFQ and serum concentrations of the carotenoids' *trans* isomers. The two methods were combined using the same approach as the present study, namely Howe's score with ranks where the number of quantiles was equal to the sample size [222], although they then modelled the risk based on a continuous scale in contrast to our quartile rankings. The study showed that both intake and biomarker levels were associated with nuclear cataracts risk and with nuclear sclerosis. In the nuclear cataract investigations, the odds ratios were stronger when using the biomarker, whereas the odds ratios were similar in the nuclear sclerosis analyses. The study also showed that, for both disease outcomes, the combination of the intake measure with the biomarker levels improved the odds ratios slightly compared to using single measures. For example, the odds for nuclear cataract were 0.77 (95%CI 0.57-1.02) for the FFQ, 0.69 (95%CI 0.51-0.94) for serum levels and 0.66 (95%CI 0.48-0.91) for the combination of dietary intake and biomarker levels. The findings of this prospective cohort study are in agreement with the results from our prospective investigations in men, showing that nutrient estimates from multiple dietary assessment methods are a stronger predictor of disease risk compared to using single nutrient estimates.

8.5.4 Strengths and limitations

The present investigations, which studied three different exposure methods as single measures or as their respective combinations, and their comparative ability to detect i) the cross-sectional associations with heel ultrasound and DXA and ii) the prospective associations with fracture risk, are completely novel. To date, only a limited number of studies have used the approach of combining different exposure measures as means of increasing the power to detect diet-disease associations [205], but to the best of my knowledge, none exists on bone health. The present results, which showed that combining different exposure measures of vitamin C in comparison to using single measures strengthened the associations with heel ultrasound in men but not in women, are thus novel findings. Moreover, our investigations comprised a large sample of 2032 and 4010 participants in the heel ultrasound study and the fracture risk study, respectively; and both studies were performed in both men and women.

The present investigations also had a number of limitations. Firstly, our analyses were based on the assumption that there is a definite relationship between vitamin C and bone health. However, despite suggestions of a number of underlying mechanisms [136, 161, 163], published epidemiological evidence is contradictory [124, 131, 166, 167, 230, 342-344], and the associations in the present cohort were also inconsistent. We propose that, in the case of a definite cross-sectional and prospective association between vitamin C and bone health, our findings would have been more consistent and may have been shown significant associations in both men and women. Another limitation was the small sample size of 151 participants in the DXA analyses. In order to allow for comparison between the heel ultrasound and DXA measurements, it was important to exclude those with missing data for either measurement, and this resulted in a very restricted cohort sample. The latter may have been too small to detect any significant associations between measures of vitamin C intake or plasma status and either bone measurement, whereas a larger sample may have had more power to show these associations.

8.6 Conclusion

The present study found that combining dietary intake and plasma estimates of vitamin C using different dietary assessment methods compared to using estimates from single measures strengthened i) the cross-sectional association between vitamin C and heel ultrasound in men only and ii) the prospective association between vitamin C and total fracture risk in men, but not in women. These findings highlight that future epidemiological studies investigating the relationship between diet and bone health should aim to estimate nutrient intakes from two different dietary assessment methods, ideally using one intake and one biomarker measure, as this could significantly increase the studies' power to detect the diet-disease relationship. Our findings are completely novel, and thus more validation studies are needed which will investigate the concept of using a combination of dietary assessment methods in association with other common chronic disease risks such as cardiovascular disease and diabetes. This will determine whether this concept is applicable to i) exposure and outcome measures other than vitamin C and bone health and ii) nutrients, where the dietary intake and the biological marker of intake do not correlate very well.

CHAPTER 9

FINAL DISCUSSION AND FUTURE DIRECTIONS

9.1 Main research findings

The purpose of this thesis was to contribute to a better understanding of the role of diet in osteoporosis and fracture prevention and to explore issues associated with the measurement of dietary intakes in populations. Current public health recommendations for the prevention of osteoporosis and fractures are limited [2], but diet may be a useful strategy as it is modifiable. A number of dietary factors have been extensively studied, and those include calcium for its role in providing bone strength and stability, vitamin D for maintaining calcium homeostasis and protein for its importance in the overall integrity of bone [3, 4, 6, 133]. However, there is only limited data on other nutrients including vitamin K_1, vitamin C and iron, despite suggestions of multiple underlying mechanisms with bone health. Vitamin K_1 may play a role in reducing bone-related inflammation [150, 151, 231], a process associated with upregulated bone resorption [232], and it is crucial to the calcium-binding ability of osteocalcin, the most abundant non-collagenous protein in bone [11]. Both vitamin C and iron play crucial cofactor roles in bone collagen synthesis [7-10], thereby increasing overall collagen strength [136]. Additionally, vitamin C may also mediate osteoclastogenesis and osteoblastogenesis [161-164]; whereas iron has another cofactor role in the synthesis of vitamin D [172, 383]. However, previous epidemiological studies, which investigated associations between these nutrients and measures of bone health, are limited with regards to studying men [155, 157, 158, 168, 182, 184, 185, 409], using data from British populations [154, 168, 186] and including nutrient status measurements as opposed to estimations of only dietary intakes [166, 169, 182, 183, 408, 409]. Moreover, the role of iron in bone health has only been studied independent of the food source [142, 181, 186], yet this does not account for the different bioavailability of animal and plant sources of iron [188-192]. A greater understanding of dietary factors, which may be beneficial to long-term bone health, could inform future diet-bone RCTs, and these data are crucial to informing future nutritional guidelines for the prevention of osteoporosis and fractures.

Establishing accurate diet-disease relationships is challenging as errors arising from the estimation of dietary intakes in populations may attenuate potential associations [200]. A number of approaches for limiting the effects of such measurement error have previously been suggested including combining measures of dietary intake with dietary biomarkers [205, 206]. The latter could provide a new strategy for improving the methodology of future nutritional epidemiological studies, thereby determining potentially more accurate associations between diet and bone health. However, only a limited number of studies have used this method and no such studies have investigated bone health [206].

This thesis aimed to i) investigate associations between dietary intakes and blood measures of a number of micronutrients with bone density from heel ultrasound measurements and fracture risk at multiple sites in men and women from the EPIC-Norfolk study, and ii) explore means of limiting the impact of measurement error on diet-disease relationships using estimates of vitamin C intake and plasma status in association with heel ultrasound and fracture risk as an example. The main findings are summarised in **Figure 9.1** and are discussed in more detail in the following sections.

Table 9.1: Summary of main research findings.

	Men					Women				
	Heel ultrasound	Fractures				Heel ultrasound	Fractures			
		Total	Hip	Spine	Wrist		Total	Hip	Spine	Wrist
Fruit and vegetable intake	×	×	✓+	×	×	✓+	×	×	×	×
Fruit intake	×	×	✓+	×	×	✓+	×	×	✓+	×
Vegetable intake	✓+	×	×	×	×	✓+	×	×	×	×
Vitamin K_1 intake	✓+	×	×	×	×	✓+	×	×	✓+	×
Vitamin C intake (diet)	✓+	✓+	×	×	×	✓+	×	×	×	×
(total)	✓+	✓+	×	×	✓+	✓+	×	×	×	×
Plasma vitamin C	×	✓+	✓+	✓+	×	×	×	×	×	×
Iron intake (diet)	×	×	×	×	×	✓+	✓+	×	✓+	×
(plant-based)	×	×	×	×	×	✓+	×	×	×	×
(animal-based)	×	×	✓−	×	×	×	×	×	✓+	×
(animal ratio)	×	✓−	✓−	×	×	×	×	×	×	×
Serum ferritin	×	×	×	×	×	×	×	×	✓+	×

The table shows the associations between a number of nutrient intakes and biomarkers with heel ultrasound (cross-sectional study) and fracture risk at multiple sites (prospective study). Dietary factors were significantly associated with higher heel ultrasound or reduced fracture risk (✓+), significantly associated with a higher fracture risk (✓−) or not associated with heel ultrasound or fracture risk (×).

9.1.1 Diet and heel ultrasound

Following the adjustment for relevant confounding factors, the results from the present cross-sectional studies suggest that higher dietary intakes of vitamin K_1 and vitamin C were significant predictors of higher VOS in men, with an effect size of 0.6% between the upper quintiles referent to the lowest quintile of intake for both nutrients, which is slightly smaller compared to the effect sizes of vegetable intake in men in this cohort (0.7%). Similarly in women, higher dietary intakes of vitamin K_1, vitamin C and iron, especially plant-based iron, were significantly associated with higher BUA. The scale of these associations between the upper and the lowest quintile in women was 3.2-5.8%, with the highest magnitude observed for plant iron intake, and this was also smaller compared to the associations with fruit and vegetable intake (5.1-7.3%) in women in this cohort. In contrast, serum ferritin concentrations as an indicator of body iron stores were not associated with heel ultrasound in women; and neither dietary iron intake nor iron status was a significant predictor of heel ultrasound in men. Moreover, there were no associations with plasma vitamin C concentrations in either sex.

Percentage differences in VOS were much smaller than those of BUA in the present study, possibly due to the scale differences between these two bone parameters. However, one previous study has shown that their relative fracture risk implications are very similar [66]. Interestingly, where significant associations were found in the present study, dietary intakes and nutrient status measurements were almost consistently associated with VOS in men and BUA in women. Only plant-based iron showed a significant positive association with both measures of heel ultrasound in women. Potential reasons for this apparent sex difference are currently not known. However, there is evidence regarding the independent heritability of the two bone parameters [77], and they have also been shown to be independently associated with osteoporotic fractures [21, 69, 70]. These hereditary properties may have affected the present cross-sectional associations between nutrient intakes and status with bone health, resulting in differing findings between BUA and VOS. To date, bone heritability has not been studied to a great extent and genetic factors have not been accounted for in most epidemiological studies as they are mostly unknown. Future research should address this as the determination of heritability-independent relationships between diet and bone health is crucial in our understanding of the role of diet in osteoporosis and fracture prevention.

To date, most epidemiological studies have used DXA scans, as it is the gold standard for measuring bone density [60], but only a limited number of studies have investigated associations between diet and ultrasound measurements [156, 290, 351]. Hence, understanding the implications of our findings is difficult, as previous data is scarce. Nevertheless, we were able to compare the effects of diet on heel ultrasound with those of age in our study by comparing the effect sizes of the extreme quintiles of nutrient intakes with a 10-year increase in age. Increasing age is a risk factor for developing both osteoporosis and fractures and it is one of the largest predictors of

low bone density [30, 31]. In women, the effect of diet on heel ultrasound in comparison to a 10-year change in age was similar, with the scale of the association for age being around two times greater than diet for BUA. In contrast to women, the effect of diet on heel ultrasound was only small in men, with the scale of the association for age being approximately 14 times larger than diet for VOS. However, dietary behaviour is modifiable; whereas ageing is not. Moreover, in both men and women, the differences between extreme quintiles of all nutrient intakes, which showed significant positive associations with heel ultrasound, are achievable through the usual diet [145, 214, 350]. Therefore, the findings from this thesis suggest that diet may be crucial to preserving higher levels of bone density in older people, especially in women; and dietary behaviour modification may be an important and feasible strategy for the prevention of osteoporosis.

9.1.2 Diet and fracture risk

The results from the present prospective studies showed that, following multivariate adjustment, higher dietary vitamin C intake was a significant predictor of 48% lower total fracture risk (hip, spine and wrist fractures combined) in men in the upper compared to the lowest quintile of intake after the median 12.6-year follow-up. In comparison to fruit and vegetable intake in men in this cohort (fruit intake: HR 0.31, 95%CI 0.15-0.65; F&V intake: HR 0.43, 95%CI 0.22-0.87), the effect size for vitamin C intake was smaller. Moreover, in men, plasma concentrations of vitamin C were an even stronger predictor of lower fracture risk than dietary intake, with a magnitude of effect between the upper and the lowest quintile of 74% at the spine, 65% at the hip and 52% for total fractures. In contrast, in men, the highest compared to the lowest intake of animal iron and the highest ratio of animal iron as a percentage of total dietary iron intake were significantly associated with higher hip fracture risk (HR 2.29, 95%CI 1.11-4.73; and HR 2.61, 95%CI 1.25-5.45, respectively). There were no associations between dietary vitamin K_1 intakes and serum ferritin concentrations with fracture risk in men. Higher dietary intakes and plasma concentrations of vitamin C may represent a higher consumption of fruit and vegetables; whereas a higher intake of animal iron may be indicative of a less healthy dietary pattern characterised by a high consumption of animal foods such as red meats at the expense of plant-based foods such as fruit and vegetables. In previous epidemiological studies, lower dietary intakes of fruit and vegetables were associated with lower BMD [90, 131, 416], and this may explain why fracture risk was increased in men with a higher animal iron intake, whereas it was lower in men with higher vitamin C intake and plasma status. In women, vitamin K_1 and vitamin C were not associated with fracture risk in the present prospective investigations. However, higher compared to the lowest dietary intake of iron was a significant predictor of up to 59% lower spine fracture risk and 35% lower total fracture risk. When iron intake was investigated by food source, we found a 56% reduction in spine fracture risk in women with the

highest compared to the lowest intakes of iron from animal sources. Moreover, serum ferritin as an indicator of body iron stores was a significant predictor of up to 70% lower spine fracture risk in women in the upper compared to the lowest quintiles. A higher intake of animal iron in women may be a reflection of the higher bioavailability of animal-based haem iron [188-192], which may be associated with more adequate iron stores. Hence, in women, high intakes of the more bioavailable iron from animal sources may play a role in fracture prevention.

A potential reason for the present sex-specific findings may be the smaller number of fracture events in men, which is consistent with the published literature [22]. Men have a lower prevalence of fractures than women after the age of 50 years [103], partly due to a higher amount of bone tissue and a shorter life span. In this case-cohort sample of EPIC-Norfolk participants, 12% of men had a fracture compared to 21% of women (P<0.001). The present investigations are likely to have had greater power to detect potential associations between diet and fracture risk in women, and we hypothesise that we may have found more significant relationships in a larger number of cases in men. Nevertheless, our prospective investigations of fracture risk were based on a case-cohort design, which addressed some of the limitations of the present dataset including the smaller prevalence of fractures in men and the unavailability of data from the full EPIC-Norfolk cohort [447].

9.1.3 Measurement error in dietary assessments

Assuming there is a positive relationship between vitamin C and bone health, we investigated whether the addition of a biomarker to an intake estimate may improve the detection and strength of the diet-disease association. The results from this study showed that combining dietary intake and plasma estimates of vitamin C using different dietary assessment methods compared to single measures strengthened the associations between vitamin C with both heel ultrasound and fracture risk in men, but not in women. For example, in men, associations between vitamin C and heel ultrasound were non-significant when using single measures, independent of the assessment method used. Although the associations remained non-significant, the combination of different measurements slightly improved the associations. For example, in comparison to the association between quartiles of plasma vitamin C and VOS in men (β±SE 0.012±0.032 m/s per quartile, P-trend=0.70), the combination of intake and blood measurements increased the β-coefficient to 0.050±0.032 m/s per quartile (P-trend=0.12) for 7dDD+plasma and to 0.016±0.031 m/s per quartile (P-trend=0.62) for 7dDD+FFQ+plasma. When we investigated the associations in a small sub-sample of participants who had additional data for DXA measurements at the hip, we found no significant associations between any of the vitamin C measures, neither as single measures nor as their respective combinations, and both heel ultrasound measurements and DXA, possibly due to the small sample size (n=151). However, in our prospective associations in men, most single or combinations of measures of

vitamin C showed significant inverse associations with fracture risk, and the strongest association was found for the combination of an intake measure with a blood concentration measure. For example, the association between plasma vitamin C and total fracture risk had a HR of 0.86 (95%CI 0.74-0.99, *P*=0.041); whereas the HR was 0.83 (95%CI 0.72-0.96, *P*=0.012) for the combination of 7dDD+plasma and 0.83 (95%CI 0.73-0.96, *P*=0.010) for FFQ+plasma.

The present investigations were based on the assumption that vitamin C intake and plasma concentrations are positively associated with bone density and inversely associated with the risk of fractures, and this was based on the biological importance of vitamin C in bone collagen synthesis [7-10]. However, previous epidemiological studies have shown inconsistent results on vitamin C and bone health, with studies reporting both significant and non-significant findings [91, 124, 133, 167, 169, 230, 341, 342, 344, 345]. A potential reason for these inconsistencies in previous study outcomes may be the use of different dietary assessment methods to estimate vitamin C intake as sources of error and bias differ between the different methods [195]. The interpretation of our findings is thus limited as the assumptions we made prior to our analyses were not confirmed in all previous studies.

9.2 Overview of strengths and limitations

The present investigations had a number of strengths and limitations. The inclusion of both men and women in the study design addressed previous limitations regarding the scarcity of data on diet and bone health in men [155, 157, 158, 168, 182, 184, 185, 409]. Moreover, the EPIC-Norfolk cohort provided more evidence for diet-bone associations in British populations, where data availability is also limited [154, 168, 186]. However, the cohort comprised almost exclusively of Caucasian participants, and thus future epidemiological studies in British populations should include a greater ethnic diversity. Another strength of the present studies was that they addressed previous limitations regarding small sample sizes [142, 156, 158, 168, 181, 183, 186, 405-408], with the present studies comprising of up to 5011 men and women. The present investigations were based on a sub-sample of the EPIC-Norfolk study as data from the whole cohort (*n*=25,639) were not available for analysis. However, as this was a random sample, this should not have affected the present findings. As shown in the post-hoc sample size calculations, significant associations between vitamin K_1 intake and heel ultrasound reached statistical significance despite the quintile sample sizes being smaller than the estimated required sample sizes, indicating the robustness of these associations. This may also apply to the associations between dietary intakes and blood markers of vitamin C and iron which were based on similar sample sizes. Nonetheless, the post-hoc sample size calculations revealed that non-significant findings of the present studies may have been a result of too small participant numbers which were not large enough to detect smaller effect sizes between upper and lower quintiles.

The present studies investigated both cross-sectional and prospective associations in the same population using different bone health measures, including heel ultrasound and fracture risk, in contrast to most previous studies which investigated only one of these parameters per population [133, 154, 159, 182, 186, 341, 405]. This allowed us to explore the associations between diet and bone health with both short-term and long-term bone health measures in this population. However, a limitation was the observational nature of the present investigations which cannot infer causality. Moreover, although multivariate adjustment models were applied in the analyses, a number of other relevant confounders previously associated with bone health, including sunlight exposure [295], were not measured as part of the EPIC-Norfolk study. Furthermore, residual confounding may have occurred despite the adjustment for covariates and may have resulted in bias in exposure effect estimates.

Another strength was that the EPIC-Norfolk study used 7dDD taken at baseline to estimate habitual dietary and supplemental intakes, and data from these were available in the present studies. The dietary analysis was based on more than 11,000 food items and almost 600 portions (DINER) [212], a previously published vitamin K_1 database [214], which had been developed further to include predominantly British food items [160, 215], and a vitamin and mineral supplement database (ViMiS) [213]. Previous validation studies on this cohort have shown that the estimated 7dDDs were most comparable to weighed food records for the majority of nutrients [202, 204]. For example, vitamin C intake estimated from a weighed food record correlated better with intake measured from the 7dDD (r=0.70) compared to the FFQ and the self-reported 24-h recall (both r=0.54). 7dDDs provide a relatively accurate indication of usual intake due to the nature of keeping a diary [195]. Moreover, as food and drinks are recorded as they are being consumed, the reliance on long-term memory is not an issue.

Despite their practicality and non-invasiveness, written dietary assessment methods including the 7dDD are likely to be subjective and prone to human recall error [170]. In contrast, biological markers of nutrient status account for factors such as length of storage of food items, cooking practises and variations in individual nutrient bioavailability which may influence actual intakes [207]. Therefore, another strength of the present investigations was the availability of nutrient status data for vitamin C (plasma vitamin C) and iron (serum ferritin) which had been measured in EPIC-Norfolk participants at baseline. However, it must be noted that any nutrient status may be influenced by a number of biological, environmental and dietary factors. For example, factors that have previously been shown to affect plasma vitamin C concentrations include age, sex, BMI, body fat distribution, fat-free mass, smoking and infection [227, 302-306].. Moreover, serum ferritin concentrations are dependent on factors including age, sex, body fat distribution and current iron stores [357, 372, 374, 375, 412-414]. Thus, neither written dietary assessment methods nor biological markers of nutrient status give an exact account of actual dietary intake;

however, the availability of both dietary measures in the present investigations allowed us to explore the diet-bone relationships from a broader perspective.

Another limitation of the present studies was that bone density was measured using ultrasound at the heel bone, which is a less precise method for determining bone density than DXA [60]. However, in the EPIC-Norfolk study, ultrasound was chosen as a non-radiative alternative method which is faster, cheaper and more portable than DXA [62]. Moreover, ultrasound has previously been shown to be capable of distinguishing bone densities of subjects with and without osteoporosis [67], and correlations between ultrasound and relevant confounders of bone density were comparable to those with DXA measurements [85]. As epidemiological studies using ultrasound measurements are scarce [156, 290], we were unable to compare some of our findings to those of previous studies, and thus the present investigations have provided a great amount of novel data, especially regarding effect sizes, for this bone density measurement.

Another limitation was the time difference between measurements in the cross-sectional study. The dietary intake estimated from the 7dDD and nutrient status measured in blood were taken at baseline, whereas the heel ultrasound measurements were performed as part of the second health examination. However, 7dDDs estimate habitual dietary intake which is likely to still be representative of intake at the time the ultrasound measurements were performed. Moreover, the rate of age-related bone loss is only small, and thus the time difference between these measurements of three years at most is unlikely to have had a significant impact on the present findings.

In the present investigations, the fracture data had been obtained from hospital admissions which are most likely underestimated for spine fractures due to a large absence in their clinical attention and radiologic detection [168, 293, 294]. This may have reduced the power of the present studies to detect the associations between nutrient intake or status and spine fracture risk, and we hypothesise that we may have had more consistent findings if we had been able to account for the underestimation of spine fractures.

9.3 Public health implications and future research directions

Current UK public health recommendations for the prevention of osteoporosis and fractures were recently updated [2] but are still limited as the underlying mechanisms of osteoporosis are still not fully understood [1]. Within these guidelines, diet-specific recommendations focus on dietary calcium intake, the use of calcium and vitamin D supplements and reduced alcohol consumption, although no specific recommendations were proposed [2]. However, the present findings have highlighted that a number of micronutrients, including vitamin K_1, vitamin C and iron, may have small but important protective effects for bone health, but further studies are

needed before these nutrients may be included in future public health guidelines for the prevention of osteoporosis and fractures. Nevertheless, this thesis addressed some previous limitations of observational studies with regards to a scarcity of data in men, in British populations, biomarkers of nutrient status and fracture risk. Although the present investigations found significant associations between vitamin K_1 intake and heel ultrasound in both men and women, there were no associations with fracture risk. Moreover, there is already a large body of evidence from RCTs on the effects of vitamin K_1 supplementation on bone health [152, 270, 274-278]. However, the present epidemiological investigations of dietary intakes and nutrient status of vitamin C and iron have provided more novel data, as these nutrients have been much less investigated in association with bone health. We found that vitamin C was a predictor of bone health predominantly in men and iron in women, with significant associations between vitamin C intake and heel ultrasound in both sexes, plasma vitamin C and fracture risk in men, iron intake and heel ultrasound in women, and both iron intake and serum ferritin with fracture risk in women. Moreover, we also found that the food source of iron played an important role in these associations. To ensure long-term bone health, our findings suggest that men should ensure adequate dietary intakes of vitamin C-rich foods such as citrus fruits and berries, whilst reducing their consumption of meat and meat products; whereas women should ensure adequate dietary intakes of iron-rich foods, especially from the more bioavailable animal sources such as red meat. Our preliminary investigations also highlighted that fruit and vegetable intake was a significant predictor of bone health in both men and women, suggesting that people should aim to follow the current international guideline of consuming at least five portions of fruit and vegetables every day [228] to ensure long-term bone health.

In terms of future work, it would be interesting to explore the potential of a synergistic relationship between vitamin C and iron in bone health, as the nutrients share a common mechanism in bone collagen synthesis [7-10]. It is well known that vitamin C and iron interact during the intestinal absorption of iron. However, nutrient interactions beyond these, such as in bone collagen synthesis, have not yet been explored. In hydroxylation reactions within bone collagen fibres, iron undergoes a cyclic oxidation and reduction which is driven by vitamin C as the reductant. The subsequent formation of covalent bonds between adjacent collagen fibres leads to stronger collagen cross-links, thus increasing overall collagen strength [136]. This may suggest that, apart from their different underlying mechanisms in bone, vitamin C and iron may also have synergistic properties which relate to the synthesis of bone collagen. However, to date, evidence is limited to the independent requirements of either nutrient in the hydroxylation reactions, and studies investigating potential interactions between vitamin C and iron in bone collagen synthesis are completely lacking. Future studies investigating this may be of observational nature and could explore whether measures of bone density are higher in people with high dietary intakes or nutrient status of both nutrients. Moreover, future studies may also

investigate this in intervention studies, looking at the effects of iron and vitamin C intake on bone collagen-specific biomarkers such as PINP, to explore whether the combination of the nutrients may improve such markers.

This thesis also explored whether combining a dietary intake estimate with a biomarker of nutrient status would improve the diet-disease association at the example of vitamin C and bone health. The present findings showed that the combined measures resulted in improved associations in men, but not in women. To the best of my knowledge, no studies have explored the combined use of dietary intake and nutrient status at the example of iron, and hence we do not know whether the findings would be comparable to our investigations of vitamin C. However, future studies aiming to explore potential synergistic relationships of vitamin C and iron in bone collagen synthesis may still consider measuring both dietary intakes and nutrient status of both nutrients, and combining vitamin C intake with plasma/serum vitamin C and iron intake with a marker of body iron stores such as serum ferritin. As we found some improvements in the diet-disease associations when combining vitamin C intake with plasma status, we think that this approach may also be useful in the detection of potential synergistic associations between vitamin C and iron in bone health.

In conclusion, this thesis has contributed to the current literature on dietary factors in osteoporosis and fracture prevention by providing novel epidemiological insights into the associations between dietary intakes and nutrient status (where available) of vitamin K_1, vitamin C and iron from different food sources with heel ultrasound measures and fracture risk at multiple sites in men and women from the EPIC-Norfolk cohort. However, further investigations are warranted, especially with regards to exploring potential synergistic effects of vitamin C and iron in bone collagen synthesis. Combining estimates of dietary intake with biomarkers of nutrient status may be a useful approach in detecting such associations in future studies.

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Appendices

Appendix 1: Literature tables for chapter 4

(Vitamin K1 & bone health)

Table A1.1: Intervention studies on vitamin K_1 and bone health included in the meta-analysis by Fang *et al.* (2012).

Study	Duration; study design	Subjects	Age (yrs)	Intervention	Primary outcome	Results*	Comments
Braam [153] 2003 Netherlands	36 months; double-blind RCT	*n:* 155 (women)	55 ± 3 (50 – 60)	3 groups: Placebo group (*n*=60): maltodextrin; Suppl. group (*n*=58): 500mg calcium, 150mg magnesium, 10mg zinc and 8µg vitamin D; K_1 group (*n*=63): same as suppl. group and 1mg phytonadione	FN, LS BMD	M	Women receiving K_1 had 1.7% and 1.3% less FN BMD loss than those taking the placebo or the supplement without vitamin K_1, respectively. No effects of vitamin K_1 supplementation on LS BMD.
					BSALP, OC	NS	No effects of vitamin K_1 supplementation on bone markers.
Braam [279] 2003 Netherlands	29 months; double-blind RCT	*n:* 115 (women) (endurance athletes)	NR (15-50)	2 groups: Placebo group (*n*=56): corn starch grain; K_1 group (*n*=59): 10mg K_1	FN, LS BMD	NS	No effects of vitamin K_1 supplementation on BMD.
					BSALP, OC, DPD	M	In the K_1 group, OC and DPD decreased by 5.6% and 2%, respectively, and this was significantly different from the placebo group (P<0.05). No significant effects on BSALP levels.
Cheung [280] 2008 Canada	24 months; double-blind RCT	*n:* 440 (women) (osteopenic)	59 ± NR (40-82)	2 groups (*receiving unspecified doses of calcium and vitamin D)*: Placebo group (*n*=202): *not specified*; K_1 group (*n*=198): 5mg K_1	TH, FN, LS, UDR BMD	NS	No effects of vitamin K_1 supplementation on BMD.
					OC, CTx	M	OC levels decreased by 16% from baseline in the K_1 group and this was significantly different from the placebo group (P<0.001). CTx levels did not differ between the groups.
Shea [272] 2008 UK	36 months; double-blind RCT	*n:* 379 (157 men; 222 women)	68 ± 6 (60-81)	2 groups *(receiving 600mg calcium and 10µg vitamin D)*: Placebo group (*n*=190): multivitamin formulation; K_1 group (*n*=189): same as placebo group + 500µg phylloquinone	FN, LS, WB BMD	NS	No effects of vitamin K_1 supplementation on BMD.
					OC, %ucOC	M	%ucOC decreased by 47% in the K_1 group; whereas it increased by 5% in the placebo group (P<0.001). No significant effects on OC levels.
Volpe [273] 2008 US	6 months; double-blind RCT	*n:* 21 (women)	36 ± 9 (25-50)	2 groups: Placebo group (*n*=10): *not specified*; K_1 group (*n*=11): 600µg phylloquinone	FN, WT, GT, LS, RS BMD	NS	No effects of vitamin K_1 supplementation on BMD.
					OC, NTx	M	NTx levels increased by around 165% in the K_1 group and this was significantly different from the 12% change in the placebo group. No significant differences in OC levels.

Reference: Fang et al. (2012) [270].

Abbreviations: TH, total hip; FN, femoral neck; GT, greater trochanter; WT, Ward's triangle; LS, lumbar spine; UDR, ultradistal radius; RS, radial shaft; WB, whole body; BSALP, bone-specific alkaline phosphatase; OC, osteocalcin; %ucOC, undercarboxylated osteocalcin as a percentage of total osteocalcin; CTx, collagen type 1 cross-linked C-telopeptide; NTx, collagen type 1 cross-linked N-telopeptide; DPD, deoxypyridinoline.

** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table A1.2: Further intervention studies on vitamin K_1 and bone health.

Study	Duration; study design	Subjects	Age (yrs)	Intervention	Primary outcome	Results*	Comments
Kruger [277] 2006 New Zealand/ UK	4 months; single-blind RCT	*n:* 82 (women)	27 ± 5 (20-35)	3 groups: Control group (*n*=26): no treatment; Calcium group (*n*=26): 1000mg calcium-enriched milk with 5µg vitamin D_3; K_1 group (*n*=26): same as calcium group + 80µg phylloquinone	OC, ucOC, CTx, PINP	S	ucOC decreased significantly by around 50% compared to baseline in K_1 group (P<0.05); in contrast to the placebo group. OC, PINP and CTx levels decreased by more than 15%, 15% and 30% respectively from baseline in both treatment groups, and values were significantly different from the placebo group (P<0.05).
Bolton-Smith [275] 2007 UK	24 months; double-blind RCT	*n:* 209 (women)	68 ± 6 (60+)	4 groups: Placebo group (*n*=56): *not specified*; K_1 group (*n*=54): 200µg K_1; Ca+D group (*n*=50): 1000mg calcium carbonate + 10µg vitamin D_3; Ca+D+K1 group (*n*=49): same as Ca+D group + 200µg K_1	FN, T, WT, MDR, UDR BMD and BMC	NS	No significant differences in BMD and BMC between groups at any bone site. Nevertheless, BMD and BMC increased from baseline by approximately 5-6 mg/cm^2 and 20 mg respectively in the Ca+D+K_1 group (P<0.05) but not in the other groups.
					BSALP, ucOC, %ucOC, NTx	M	ucOC levels decreased by 31% and 45% and %ucOC decreased by 48% and 54% for the the K_1 group and the Ca+D+K_1 group respectively compared to baseline levels (P<0.001); whereas ucOC levels increased by up to 69% in the placebo group and the Ca+D group (P<0.001). No significant differences in BSALP and NTx between groups.
Bügel [276] 2007 Denmark/ Netherlands/ Ireland	3x 1.5 months; double-blind cross-over RCT	*n:* 31 (women)	63 ± 4 (NR)	3 cross-over treatments *(subjects also received 10µg vitamin D_3)*: Control: no treatment; Low K_1: 200µg phylloquinone; High K_1: 500µg phylloquinone	BSALP, OC, ucOC, NTx, PYR, DPD	M	In comparison to the control group, ucOC levels decreased by around 40% and 68% with 200 and 500 µg vitamin K_1, respectively (P<0.001). The 500 g K_1 supplementation also resulted in approximately 15% higher OC levels compared to the control (P<0.05). No significant effects on any other bone turnover marker.
Booth [278] 2008 US	36 months; double-blind RCT	*n:* 401 (164 men, 237 women)	69 ± 6 (60-80)	2 groups *(receiving 600mg calcium carbonate and 10µg vitamin D_3)*: Control group (*n*=223): unspecified multivitamin K1 group (*n*=229): same as control group + 500µg phylloquinone	FN, LS, WB BMD	NS	No significant differences in BMD changes between groups at any bone site.
					OC, %ucOC, NTx	M	The K_1 group had 44-52% lower %ucOC levels compared to baseline (P<0.001); whereas there were no changes in the control group. No significant effects on serum NTX and OC.
Binkley [274] 2009 US	12 months; double-blind RCT	*n:* 329 (women)	62 ± 1 (NR)	3 groups *(receiving 315mg calcium and 5µg vitamin D_3)*: Control group (*n*=115): 1x placebo phylloquinone + 3x placebo MK4; K_1 group (*n*=108): 1mg phylloquinone + 3x placebo MK4; K_2 group (*n*=106): 1x placebo phylloquinone + 3x 15mg MK4	TH, LS BMD	NS	No effects of vitamin K_1 supplementation on BMD at any site.
					BUA, SOS	NS	No effects of vitamin K_1 supplementation on BUA or SOS.
					BSALP, OC, ucOC , NTx	M	The percentage difference between the K_1 group and the control group following the intervention was -8% for OC and 61% for ucOC (P≤0.005). No significant effects on BSALP and NTX.

Table A1.2: (continued)

Study	Duration; study design	Subjects	Age (yrs)	Intervention	Primary outcome	Results*	Comments
Kanellakis [152] 2012 Greece/ Netherlands	12 months; RCT	*n:* 115 (women)	62 ± 6 (54-73)	4 groups *(treatment given in form of dairy foods)*: Control group (*n*=39): no treatment; Ca+D group (*n*=26): 800mg calcium + 10*μg* vitamin D_3; K_1 group (*n*=26): Same as Ca+D group + 100μg phylloquinone; K_2 group (*n*=24): Same as Ca+D group + 100μg menaquinone-7	TH, FN, T, iT, WT, LS, WB, pelvis, leg, arm BMD	M	The Ca+D group, K_1 group and K_2 group had significantly higher WB BMD (2.1%, 1.1% and 1.1% respectively) compared to baseline (*P*<0.05), and this was significantly different from the control group (-0.1%, *P*=0.001). Changes in LS BMD also differed significantly in the K_1 group (1.35%) and the K_2 group (0.5%) compared to the control group (-2.9%; *P*=0.001). No significant differences at other BMD sites.
					OC, %ucOC, PYR, DPD	M	%ucOC levels in the K_1 group and K_2 group reduced from 40.8% and 47% at baseline to 27.5% and 23.4% at 12 months; whereas %ucOC levels increased in the Ca+D group and the control group (*P*<0.001). DPD was also borderline significantly lower in the K_1 group (9.9%) and K_2 group (9.8%) compared to the Ca+D group (12.6%) and the control group (11.1%; *P*=0.047). No significant differences for other bone markers between groups.

Abbreviations: TH, total hip; FN, femoral neck; T, trochanter; iT, intertrochanter; WT, Ward's triangle; LS, lumbar spine; MDR, mid-distal radius; UDR, ultradistal radius; WB, whole body; SOS, speed of sound; BSALP, bone-specific alkaline phosphatase; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; CTx, collagen type 1 cross-linked C-telopeptide; NTx, collagen type 1 cross-linked N-telopeptide; PINP, procollagen type I N-terminal propeptide; PYR, pyridinoline; DPD, deoxypyridinoline; MK4, menatetrenone.

** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table A1.3: Prospective studies on vitamin K_1 and bone health.

Study	Follow-up	Subjects	Age (yrs)	Dietary assessment	Mean ± SD (range) vitamin K_1 intake or blood levels	Outcome measures and analyses	Results*	Comments
Szulc [236] 1996 France	3 yrs	*n*: 183 (women) *(n=30 with fracture (F); n=153 without fracture (NF))*	86 ± 7 (F) 83 ± 6 (NF) (70-97)	N/A	**OC levels:** F = 7.9±4.3 ng/ml **;** NF = 6.2±3.3 ng/ml **ucOC levels:** F = 1.5±1.7 ng/ml **;** NF = 0.9±0.9 ng/ml **ALP levels:** F = 92±40 IU/l**;** NF = 78±37 IU/l	Risk of hip fracture by OC, ucOC and ALP levels	Status: S	Women who sustained a hip fracture during the 3-year follow-up had significantly higher serum OC (27%), ucOC (67%) and ALP levels (18%) than those without a fracture (*P*<0.05).
Feskanich [159] 1999 US	10 yrs	*n*: 72,327 (women)	51 ± 7 (38-63)	FFQ	**Dietary intake:** Median = 163 µg/d Quintile 1 < 109 µg/d Quintile 2 = 109-145 µg/d Quintile 3 = 146-183 µg/d Quintile 4 = 184-242 µg/d Quintile 5 > 242 µg/d	Risk of hip fracture by quintiles of vitamin K_1 intake	Diet: S	When quintiles 2-5 were combined, women in the upper quintiles had a reduced risk of a hip fracture compared to those in quintile 1 (RR 0.70, 95%CI 0.53-0.93).
Booth [158] 2000 US	7 yrs	*n*: 888 (335 men; 553 women)	75 ± 5 (68-94)	FFQ	**Dietary intake:** Men = 143±97 µg/d Women = 163±115 µg/d Quartile 1 = 56 µg/d (median) Quartile 2 = 105 µg/d Quartile 3 = 156 µg/d Quartile 4 = 254 µg/d	Change in hip, spine and radial BMD by quartiles of vitamin K_1 intake	Diet: NS	No significant associations between vitamin K_1 intake and changes in BMD over 7 years in men and in women.
						Risk of hip fracture by quartiles of vitamin K_1 intake	Diet: S	In the combined sample, those in quartile 4 had a significantly reduced risk of hip fractures compared to quartile 1 (RR 0.35, 95%CI 0.13-0.94), and higher vitamin K_1 intakes were borderline significantly associated with lower hip fracture risk (*P*-trend=0.047).
Rejnmark [281] 2006 Denmark	5 yrs	*n*: 1139 – 1869 (women)	50 (median) (43-58)	4dDD or 7dDD	**Dietary intake at baseline:** Quartile 1 < 46 µg/d Quartile 2 = 46-67 µg/d Quartile 3 = 67-105 µg/d Quartile 4 > 105 µg/d Lowest 5% of intake <24.5 µg/d Highest 5% of intake >209 µg/d **Intake at 5-year follow-up:** Quartile 1 < 38 µg/d Quartile 2 = 38-60 µg/d Quartile 3 = 60-99 µg/d Quartile 4 > 99 µg/d Lowest 5% of intake <17 µg/d Highest 5% of intake >214 µg/d	Changes in FN and LS BMD by quartiles of vitamin K_1 intake	Diet: NS	No significant associations between quartiles of vitamin K_1 intake and BMD between baseline and 5-yr follow-up (baseline intake) and between 5-yr and 10-yr follow-up (intake at 5-yr follow-up).
						Changes in FN and LS BMD between lowest and highest 5% of vitamin K_1 intake	Diet: NS	No significant differences in changes in BMD over 5 years at either site between the extreme 5% of vitamin K_1 intake.

Table A1.3: (continued)

Study	Follow-up	Subjects	Age (yrs)	Dietary assessment	Mean ± SD (range) vitamin K_1 intake or blood levels	Outcome measures and analyses	Results*	Comments
Tsugawa [284] 2008 Japan	3-4 yrs	*n*: 379 (women)	63 ±11 (30-88)	N/A	**Plasma vitamin K_1:** 1.58±1.2 ng/ml Low K_1 group < 1.20 ng/ml High K_1 group > 1.20 ng/ml	Vertebral fracture incidence by plasma K_1 or ucOC	Status: M	Plasma K_1 concentrations were significantly negatively associated with the incidence of vertebral fractures (β -0.244; *P*=0.007). No significant association with ucOC.
					ucOC levels: 4.68±3.2 ng/ml	Relative risk of vertebral fractures in low and high plasma K_1 groups	Status: S	The RR for vertebral fractures in the low K_1 group was 3.58 (95%CI 3.26-3.93) compared to the high K_1 group.
Apalset [157] 2011 Norway	10 yrs	*n*: 2807 (1238 men; 1569 women)	72 ± 1 (71-75)	FFQ	**Dietary intake:** Men: Quartile 1 < 52.9 µg/d Quartile 2 = 52.9-77.4 µg/d Quartile 3 = 77.4-113.9 µg/d Quartile 4 > 113.9 µg/d Women: Quartile 1 < 42.2 µg/d Quartile 2 = 42.2-66.7 µg/d Quartile 3 = 66.8-108.6 µg/d Quartile 4 > 108.7 µg/d	Risk of hip fracture stratified by 10 µg/d increments in vitamin K_1 intake	Diet: S	Every 10 µg/d increment in dietary vitamin K_1 intake was borderline significantly associated with a 2% reduction in hip fracture risk (HR 0.98, 95%CI 0.95-1.00; *P*=0.030).
						Risk of hip fracture by quartiles of vitamin K_1 intake	Diet: S	In the combined sample, the risk for hip fracture was higher for those in quartiles 1 and 2 compared to quartile 4 (quartile1: HR 1.63, 95%CI 1.06-2.49; quartile 2: HR 1.21, 95%CI 0.82-1.80; *P*-trend=0.015).
Bullo [156] 2011 Spain	2 yrs	*n*: 200 (men; women, *n not reported*)	67 ± 6 (55-80)	FFQ	**Dietary intake:** Men = 333.6±17.3 µg/d Women = 299.8±11.6 µg/d	Change in BUA and SOS by change in vitamin K_1 intake	Diet: NS	No significant differences in changes in BUA/SOS during follow-up between subjects who either increased or decreased their dietary vitamin K_1 intake during the follow-up.
Chan [282] 2011 Hong Kong	4 yrs	*n:* 2217 (1225 men; 992 women)	72 ± 5 (65+)	FFQ	**Dietary intake:** Men = 248.0 µg/d (median); (161.8-365.9 µg/d) Women = 242.9 µg/d (median); (169.1-343.5 µg/d)	Hip and FN BMD loss by vitamin K_1 intake	Diet: NS	Vitamin K_1 intake was not associated with hip and FN BMD loss after the 4-year follow-up.
Chan [283] 2012 Hong Kong	6.9 yrs	*n:* 2944 (1605 men; 1339 women)	74 ± 5 (NR)	FFQ	**Dietary intake:** Men: 240.9-266.7 µg/d (medians); (154.8-361.9 µg/d)† Women: 238.8-244.0 µg/d (medians); (161.9-407.9 µg/d)†	Vitamin K_1 intake in subjects with and without fracture	Diet: NS	Vitamin K_1 intake did not differ between subjects with and without a hip or non-vertebral fracture.
						Fracture risk by vitamin K_1 intake	Diet: NS	Vitamin K_1 intake was not associated with hip and non-vertebral fracture risk.

Abbreviations: FN, femoral neck; LS, lumbar spine; ALP, alkaline phosphatase; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; SOS, speed of sound.

** Results were significant (S), non-significant (NS) or of mixed nature (M).*

† Vitamin K1 intake was reported separately for participants with and without hip fracture, and with and without vertebral fracture.

Table A1.4: Case-control studies vitamin K_1 intake or status in osteoporosis and fracture patients.

Study	Subjects	Age (yrs)	Dietary assessment	Mean ± SD or range vitamin K_1 intake or blood levels	Outcome measures and analyses	Results*	Comments
Hodges [289] 1993 France	*n*: 51 hip fracture cases; 38 controls; (women)	81 ± NR (NR)	N/A	**Serum vitamin K_1:** CA = 0.34±0.3 ng/ml; CO = 0.59±0.5 ng/ml	Serum vitamin K_1 levels in age-matched cases and controls	Status: S	Cases had significantly lower serum vitamin K_1 levels (42%) than controls (P<0.01).
Kanai [288] 1997 Japan	*n*: 19 low BMD cases; 52 controls; (women)	54 ± 6 (NR)	N/A	**Serum vitamin K_1:** CA = 0.41±0.2 ng/ml; CO = 0.64±0.4 ng/ml	Serum vitamin K_1 levels in cases and controls	Status: S	Cases had significantly lower serum vitamin K_1 levels (36%) than controls (P<0.05).
Vergnaud [235] 1997 France/ Japan	*n*: 104 hip fracture cases; 255 controls; (women)	82 ± 4 (75+)	N/A	**Serum OC:** CA = 28.0±12.5 ng/ml; CO = 27.5±11.2 ng/ml **Serum ucOC (ELISA or HAP):** CA = 6.7±4.8 ng/ml; CO = 5.8±4.1 ng/ml CA = 5.1±3.7 ng/ml; CO = 4.4±3.3 ng/ml **%ucOC (HAP):** CA = 17.0±7.5 %; CO = 15.1±6.8 %	Serum OC, ucOC measured by two different techniques or %ucOC in cases and controls	Status: M	%ucOC was significantly higher in cases than in age-matched controls (17.0±7.5 *vs*. 15.1±6.8, P=0.04). Serum OC and ucOC measured by either technique did not differ between cases and controls.
Tamatani [287] 1998 Japan	*n*: 12 osteopenic cases; 15 controls; (men)	74 ± 10 (60-90)	N/A	**Plasma vitamin K_1:** CA = 0.27±0.3 ng/ml; CO = 0.38±0.3 ng/ml	Plasma vitamin K_1 levels in cases and controls	Status: S	Cases had significantly lower plasma vitamin K_1 levels (29%) than controls (P<0.05).
Rejnmark [281] 2006 Denmark	*n*: 360 fracture cases; 1140 controls; (women)	50 (median) (48-52)	4dDD or 7dDD	**Vitamin K_1 intake:** CA = 66 µg/d; CO = 67 µg/d (median) Quartile 1 < 46 µg/d Quartile 2 = 46-67 µg/d Quartile 3 = 68-105 µg/d Quartile 4 > 105 µg/d Lowest 5% of intake <25 µg/d Highest 5% of intake >210 µg/d	Differences in FN and LS BMD between cases and controls	Diet: S	Cases compared to controls had significantly lower FN BMD (median: 0.758 *vs.* 0.795 g/cm^2, P<0.001) and LS BMD (median: 0.981 *vs.* 1.033 g/cm^2, P<0.001).
					Fracture risk in cases and controls by quartiles of vitamin K_1 intake	Diet: NS	No significant differences in the risk of fractures between cases and controls by vitamin K_1 intake.
					Fracture risk in lowest and highest 5% of K_1 intake	Diet: NS	No significant differences in the risk of fractures between cases and controls in the extreme 5% of vitamin K_1 intake.
Nakano [286] 2011 Japan	*n*: 99 hip fracture cases; 48 controls (40 men; 107 women)	83 ± 8 (NR)	N/A	**Plasma vitamin K_1 (men; women):** CA = 0.31±0.2 ng/ml; CO = 0.55±0.3 ng/ml CA = 0.46±0.4 ng/ml; CO = 0.77±0.4 ng/ml	Plasma vitamin K_1 levels in cases and controls	Status: S	Cases had significantly lower plasma vitamin K_1 levels than controls in both men (44%, P<0.05) and women (40%, P<0.01).

Table A1.4: (continued)

Study	Subjects	Age (yrs)	Dietary assessment	Mean ± SD or range vitamin K_1 intake or blood levels	Outcome measures and analyses	Results	Comments
Torbergson [285] 2014 Norway	*n:*111 hip fracture cases; 73 controls (53 men; 131 women)	83 ± 9 (NR)	N/A	**Serum vitamin K_1:** CA = 0.24±0.3 ng/ml; CO = 0.55±0.6 ng/ml **ucOC levels:** CA = 2.3±3.5 ng/ml; CO = 2.7±5.4 ng/ml	Serum vitamin K_1 levels and ucOC levels in cases and controls	Status: M	Cases had significantly lower serum vitamin K_1 levels (56%) than controls (*P*<0.001). No differences in ucOC between cases and controls.
					Hip fracture risk by serum vitamin K_1 levels and ucOC levels	Status: S	Low serum vitamin K_1 levels were a significant predictor of hip fracture risk (OR 0.07, 95%CI 0.02-0.32; *P*=0.001). No associations with ucOC.

Abbreviations: CA, cases; CO, controls; FN, femoral neck; LS, lumbar spine; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; %ucOC, undercarboxylated osteocalcin as a percentage of total osteocalcin.

** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table A1.5: Cross-sectional studies on vitamin K_1 and bone health.

Study	Subjects	Age (yrs)	Dietary assessment	Mean ± SD (range) vitamin K_1 intake or blood levels	Outcome measures and analyses	Results*	Comments
Szulc [237] 1994 France	*n*: 98 (women)	81 ± 6 (NR)	N/A	**ucOC levels:** Normal <1.65 ng/ml High > 1.65 ng/ml	Hip BMD (FN, T, iT, WT) in women with normal and high ucOC levels	Status: M	Women with elevated compared to normal ucOC levels had significantly lower BMD at the total hip, FN and T sites (e.g. for FN: 0.58±0.13 *vs.* 0.43±0.13 g/cm^2, *P*<0.001).
Booth [158] 2000 US	*n*: 888 (335 men; 553 women)	75 ± 5 (68-94)	FFQ	**Dietary intake:** Men = 143±97 µg/d Women = 163±115 µg/d Quartile 1 = 59 µg/d (median) Quartile 2 = 106 µg/d Quartile 3 = 159 µg/d Quartile 4 = 248 µg/d	Hip (FN, T, WT), LS and radial (R, UDR) BMD by quartiles of vitamin K_1 intake	Diet: NS	No significant associations between vitamin K_1 intake and any BMD site in men and in women.
Booth [155] 2003 US	*n*: 2591 (1112 men; 1479 women)	59 ± 9 (29-86)	FFQ	**Dietary intake:** Men: Mean = 153±115 µg/d Quartile 1 = 8-87 µg/d Quartile 2 = 88-129 µg/d Quartile 3 = 130-189 µg/d Quartile 4 = 190-1956 µg/d Women: Mean = 171±103 µg/d Quartile 1 = 13-101 µg/d Quartile 2 = 102-148 µg/d Quartile 3 = 149-216 µg/d Quartile 4 = 217-983 µg/d	Hip (FN, T, WT) and LS BMD by quartiles of vitamin K_1 intake	Diet: M	Higher vitamin K_1 intakes were associated with higher BMD at all sites in women (*P*-trend≤0.005), for example those in quartile 4 compared to quartile 1 had 4.0%, 3.6%, 5.8% and 4.4% higher BMD at the FN, T, WT and LS, respectively. No significant associations in men.
Booth [234] 2004 US	*n*: 1604 (741 men; 863 women)	59 ± NR (32-86)	FFQ	**Men:** K_1 intake = 151±119 µg/d Plasma K_1 = 0.69±0.9 ng/ml %ucOC = 16.1±16.2 % **Premp. women:** K_1 intake = 172±104 µg/d Plasma K_1 = 0.47±0.5 ng/ml %ucOC = 17.6±16.7 % **Postmp. women (oestrogen / no oestrogen):** K_1 intake = 177±101 / 164±92 µg/d Plasma K_1 = 0.66±0.6 / 0.64±0.7 ng/ml %ucOC = 14.3±15.9 / 23.5±18.4 %	Hip (FN, T) and LS BMD by plasma vitamin K_1 or %ucOC	Status: M	In men, low plasma K_1 concentrations were associated with low hip BMD (β 0.006-0.007 g/cm^2; *P*≤0.05), as was high serum %ucOC (β -0.0008-(-)0.001 g/cm^2; *P*≤0.01). No associations between plasma K_1 and LS BMD in men. In postmenopausal women not taking oestrogen, there was a significant association between low plasma K_1 levels and low LS BMD (β 0.015 g/cm^2; *P*=0.007). No significant associations for postmenopausal women taking oestrogen and for premenopausal women.

Table A1.5: (continued)

Study	Subjects	Age (yrs)	Dietary assessment	Mean ± SD (range) vitamin K_1 intake or blood levels	Outcome measures and analyses	Results*	Comments
McLean [290] 2006 US	*n*: 1351 (583 men; 768 women)	59 ± NR (NR)	FFQ	**Men:** K_1 intake = 151±120 µg/d Plasma K_1 = 0.68±0.9 ng/ml %ucOC = 16.2±16.2 % **Premp. women:** K_1 intake = 165±96 µg/d Plasma K_1 = 0.50±0.5 ng/ml %ucOC = 18.2±16.8 % **Postmp. women (oestrogen / no oestrogen):** K_1 intake = 176±102 / 164±92 µg/d Plasma K_1 = 0.68±0.6 / 0.63±0.7 ng/ml %ucOC = 14.5±15.9 / 23.6±18.4 %	BUA and SOS by vitamin K_1intake	Diet: NS	No significant associations between vitamin K_1 intake and both BUA and SOS in men and in women.
					BUA and SOS by plasma vitamin K_1 levels or %ucOC	Status: M	In men, each 1 nmol/l (0.4507 ng/ml) increase in plasma vitamin K_1 was associated with an increase in BUA of 1.13 dB/MHz and in SOS of 1.6 m/s (*P*=0.02). %ucOC was not associated with either bone parameter in men. No significant associations in women.
Rejnmark [281] 2006 Denmark	Dataset 1: *n*: 1869 (women)	50 (median) (43-58)	4dDD or 7dDD	**Dietary intake (Dataset 1):** Quartile 1 < 46 µg/d Quartile 2 = 46-67 µg/d Quartile 3 = 67-105 µg/d Quartile 4 > 105 µg/d Lowest 5% of intake <24.5 µg/d Highest 5%of intake >209 µg/d **Dietary intake (Dataset 2):** Quartile 1 < 38 µg/d Quartile 2 = 38-60 µg/d Quartile 3 = 60-99 µg/d Quartile 4 > 99 µg/d Lowest 5% of intake <17 µg/d Highest 5%of intake >214 µg/d	FN and LS BMD by quartiles of vitamin K_1 intake	Diet: NS	No significant associations between vitamin K_1 intake and BMD in either dataset.
	Dataset 2: *n*: 1139 (women)	55 (median) (48-63)			Differences in FN and LS BMD between lowest and highest 5% of vitamin K_1 intake	Diet: NS	No significant differences in BMD between the extreme 5% of vitamin K_1 intake in either dataset.
Macdonald [154] 2008 UK	*n*: 2466 - 3199 (women)	49 ± 2 (49-54)	FFQ	**Dietary intake:** Mean = 107±50 µg/d (8-494 µg/d) Quartile 1 = 59±17 µg/d (mean) Quartile 2 = 91±16 µg/d Quartile 3 = 116±19 µg/d Quartile 4 = 162±57 µg/d	FN and LS BMD by quartiles of vitamin K_1 intake	Diet: M	A borderline significant positive association between vitamin K_1 intake and FN BMD (*P*-trend=0.044), for example FN BMD was 1.4% and 0.4% higher in quartiles 3 and 4 compared to 1. No association with LS BMD.
					PINP levels by quartiles of vitamin K_1 intake	Diet: NS	No significant associations between vitamin K_1 and PINP levels.
					DPYD/Cr and PYD/Cr ratios by quartiles of vitamin K_1 intake	Diet: S	The ratios of PYD/Cr and DPYD/Cr were both 5.6% lower in quartile 4 compared to quartile 1 (*P*≤0.002; *P*-trend=0.003).

Table A1.5: (continued)

Study	Subjects	Age (yrs)	Dietary assessment	Mean ± SD (range) vitamin K_1 intake or blood levels	Outcome measures and analyses	Results*	Comments
Apalset [157] 2011 Norway	*n:* 4461 (1886 men; 2575 women)	49 ± 1 (47-50) ; 73 ± 1 (71-75)	FFQ	**Dietary intake (47-50 yrs):** Men = 129.1±119.2 µg/d Women = 132.4±116.0 µg/d **Dietary intake (71-75 yrs):** Men = 101.1±78.7 µg/d Women = 97.0±91.4 µg/d	Risk of low BMD by quartiles of vitamin K_1	Diet: M	In the age-combined sample, in women, lower vitamin K_1 intakes were significantly associated with a higher risk of having low BMD (*P*-trend=0.007); and women in quartile 1 compared to quartile 4 had a significantly higher risk of low BMD (OR 1.60, 95%CI 1.14-2.24). No associations in men.
Bullo [156] 2011 Spain	*n*: 362 (162 men; 200 women)	67 ± 6 (55-80)	FFQ	**Dietary intake:** Men = 333.6±17.3 µg/d Women = 299.8±11.6 µg/d	BUA and VOS by 100µg/d increments in vitamin K_1 intake	Diet: S	A 100 µg/d increment in vitamin K_1 intake was associated with an increase of 0.96 dB/MHz in BUA (*P*=0.039) and of 1.13 m/s in VOS (*P*=0.028).
	n: 125 (64 men; 61 women)			**Dietary intake:** *(Data not shown)*	BSALP and DPD/CR by 100µg/d increments in vitamin K_1 intake	Diet: NS	No significant associations between vitamin K_1 intake and bone markers.
Emaus [233] 2013 Norway/ Australia	*n:* 285-334 (women)	54 ± 3 (50-60)	N/A	**ucOC levels:** 4.12±2.6 ng/ml **%ucOC:** 18.8±10.6 %	TH, FN, LS and WB BMD by ucOC and %ucOC	Status: M	Higher ucOC levels were significantly associated with lower BMD at all sites (β±SE: TH and FN: -0.007±0.002 g/cm^2, *P*≤0.008; LS: -0.008±0.003 g/cm^2, *P*=0.008; WB: -0.005±0.002 g/cm^2, *P*=0.003). In contrast, no associations were found for %ucOC.

Abbreviations: FN, femoral neck; T, trochanter; iT, intertrochanter; WT, Ward's triangle; LS, lumbar spine; R, radius; UDR, ultradistal radius; SOS, speed of sound; BSALP, bone-specific alkaline phosphatase; OC, osteocalcin; ucOC, undercarboxylated osteocalcin; %ucOC, undercarboxylated osteocalcin as a percentage of total osteocalcin; PINP, procollagen type I N-terminal propeptide; PYD/Cr, free pyridinoline cross-links relative to creatinine; DPYD/Cr, free deoxypyridinoline cross-links relative to creatinine.

** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Appendix 2: Literature tables for chapter 5

(Vitamin C & bone health)

Table A2.1: Intervention studies

Table A2.2: Prospective studies

Table A2.3: Case-control studies

Table A2.4: Cross-sectional studies

Table A2.1: Intervention studies on vitamin C and bone health.

Study	Duration; study design	Subjects	Age (yrs)	Intervention	Primary outcome	Results*	Comments
Maimoun [333] 2008 France	2 months; /	*n* 13 (4 men, 9 women)	NR ± NR (69 - 79)	No groups. All participants received the following treatment: 60 min of aerobic exercise 3 times/wk, vitamin C (500 mg/d) & vitamin E (100 mg/d)	Markers of calcium homeostasis, BSALP, OC and CTx	M	A significant increase in serum vitamin D and decrease in intact parathyroid hormone *(Details not reported)*. BSALP concentration decreased significantly by 14.5% (P=*Data not reported*). Differences in OC levels (2.3%) and CTx levels (8.8%) were not significant.
Chuin [332] 2009 Canada/France	6 months; randomised, controlled pilot study	*n* 34 (women)	NR ± NR (61 - 73)	4 groups. Placebo group (*n* 7): placebo (lactose); Vitamin group (*n* 8): ascorbic acid (1,000 mg/d) & α-tocopherol (600 mg/d); Exercise & placebo group (*n* 11): 60 min of resistance training 3 times/wk & placebo in form of lactose; Exercise & vitamin group (*n* 8): 60 min of resistance training 3 times/wk & ascorbic acid (1,000 mg/d) & α-tocopherol (600 mg/d)	FN and LS BMD	M	LS BMD decreased significantly by 1% in the placebo group (BMD pre: 1.01 ± 0.17 g/cm^2; BMD post: 1.00 ± 0.16 g/cm^2; P<0.05) but remained stable in the three intervention groups. No significant differences in FN BMD between the groups.
Ruiz-Ramos [331] 2010 Mexico	12 months; double-blind RCT	*n* 90 (25 men, 65 women)	68 ± NR (NR)	3 groups: Placebo group (*n* 30): placebo *(no details)*; Low vitamin group (*n* 30): ascorbic acid (500 mg/d) & α-tocopherol (400 IU/d); High vitamin group (*n* 30): ascorbic acid (1000 mg/d) & α-tocopherol (400 IU/d)	TH and LS BMD	M	The high vitamin group lost significantly less bone at the hip compared to the placebo group *(Details not reported)*. No significant differences in LS BMD between groups.

Abbreviations: BSALP, alkaline phosphatase; OC, osteocalcin; CTx, collagen type 1 cross-linked C-telopeptide; FN, femoral neck; LS, lumbar spine; TH, total hip.
** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table A2.2: Prospective studies on vitamin C and bone health.

Study	Follow-up	Subjects	Age (yrs)	Dietary assessment	Vitamin C intake (mg/d)*	Outcome measures and analyses	Results†	Comments
Kaptoge [166] 2003 UK	2-5 yrs	*n* 944 (470 men; 474 women)	72 (67-79)	7dDD	**Median (range) dietary intake:** Tertile 1 = 73 (7-57) Tertile 2 = 78 (58-98) Tertile 3 = 132 (99-363) *Plasma data not shown.*	2-5 year change in TH BMD stratified by tertiles of either dietary vitamin C intake or plasma vitamin C levels	Diet: M Plasma: NS	Women in tertile 2 and 3 of vitamin C intake had around 52% and 54% less hip BMD loss, respectively (*P*=0.015 and *P*=0.010; *P*-trend=0.016). No associations with intake in men and no associations between plasma and change in TH BMD in either sex.
Sahni [230] 2008 US	4 yrs	*n* 606 (213 men; 393 women)	75	FFQ	**Mean (SD) dietary intake:** Men = 141 (73) Women = 158 (83) Mean (SD) suppl. intake: Men = 82 (235) Women = 95 (248) Mean (SD) total intake: Men = 223 (259) Women = 253 (267) *Intake data for tertiles not shown.*	4-year change in LS, FN, T and RS BMD stratified by tertiles of dietary or total vitamin C intake or categories of suppl. vitamin C intake and either calcium intake, vitamin E intake, smoking or oestrogen use	Diet: M Suppl.: NS	In men, LS and T BMD loss was significantly less with higher dietary vitamin C intakes (*P*-trend≤0.05). FN and T BMD loss was significantly less for higher total vitamin C intake among men with low calcium intakes or low total vitamin E intakes (*P*-trend≤0.03). A reduction in T BMD loss of around 102% was found between tertile 3 *vs.* 1 of total vitamin C intake among men with low calcium intakes (*P*<0.05). No significant associations for suppl. vitamin C intake in men. No significant associations in women.
Sahni [167] 2009 US	15-17 yrs	*n* 918 (39.1% men; 60.9% women)	75	FFQ	**Median (range) dietary intake:** Tertile 1 = 86 Tertile 2 = 133 Tertile 3 = 208 Suppl. intake: Tertile 1 = 0 Tertile 2 < 75 Tertile 3 ≥ 75 Median (range) total intake: Tertile 1 = 94 / 95§ Tertile 2 = *Data not shown* Tertile 3 = 313 / 308§	Risk of hip fracture or non-vertebral fracture stratified by tertiles of dietary, suppl. or total vitamin C intake in the combined sample of men and women	Diet: NS Suppl.: M Total: M	A reduction in hip fracture of 69% for tertile 3 compared to tertile 1 of supplemental vitamin C intake (*P*=0.007; *P*-trend=0.02) and of 44% for total vitamin C intake (*P*=0.04; *P*-trend=0.04). No significant associations with non-vertebral fractures and no associations between dietary vitamin C intake and fracture risk at any site (*P*-trend=*Data not shown*).

Abbreviations: TH, total hip; Suppl, supplement; LS, lumbar spine; FN, femoral neck; T, trochanter; RS, radial shaft.

** Total intake is the sum of dietary intake and intake from supplements.*

† Results were significant (S), non-significant (NS) or of mixed nature (M).

Table A2.3: Case-control studies of vitamin C intake or status in osteoporosis and fracture patients.

Study	Subjects	Age (yrs)	Dietary assessment	Mean ± SD or range vitamin C intake or blood levels	Outcome measures and analyses	Results	Comments
Falch [338] 1998 Norway	*n* 40 hip fracture cases; 102 controls (men and women)	82 (men) 83 (women)	N/A	**Serum concentrations:** CA = 37 µmol/L, CO = 50 µmol/L Serum concentrations in 20 case-control pairs matched for age: CA = 34 µmol/L, CO = 54 µmol/L	Serum vitamin C concentrations in cases and controls or in 20 case-control pairs matched for age	Serum: S	Serum vitamin C concentrations were significantly lower in cases than in controls (P<0.01).
Lumbers [339] 2001 UK	*n* 75 hip fracture cases; 50 controls (women)	80 (61-103)	three 24hRs	**Dietary intake:** CA = 60.7 mg/d, CO = 55.2 mg/d Plasma concentrations: CA = 42.7 µmol/L, CO = 20.8 µmol/L	Vitamin C intakes or plasma concentrations in cases and controls	Intake: NS Plasma: S	No significant differences between vitamin C intakes in cases and controls; however, plasma concentrations were significantly higher in cases than in controls (P<0.001).
Maggio [337] 2003 Italy	*n* 75 osteoporotics; 75 controls (women)	60+	N/A	**Plasma concentrations:** CA = 30.0 µmol/L, CO = 55.5 µmol/L	Plasma vitamin C concentrations in cases and controls	Plasma: S	Cases had significantly lower plasma vitamin C concentrations than controls (P<0.001).
Martinez-Ramirez [336] 2007 Spain	*n* 167 fracture cases; 167 controls (20% men; 80% women)	65+	FFQ	**Intake:** CA = 268 mg/d, CO = 275 mg/d Quartile 1 ≤ 203 mg/d Quartile 2 = 204-247 mg/d Quartile 3 = 248-334 mg/d Quartile 4 > 334 mg/d Serum concentrations: CA = 17.6 µmol/L, CO = 23.3 µmol/L Quartile 1 ≤ 8.4 µmol/L Quartile 2 = 8.5-19.6 µmol/L Quartile 3 = 19.7-34.1 µmol/L Quartile 4 > 34.1 µmol/L	Vitamin C intakes or serum concentrations in cases and controls and in association with fracture risk	Intake: M Serum: S	No significant differences in mean vitamin C intakes between cases and controls apart from a marginal significant fracture risk reduction for quartile 2 compared to quartile 1 of vitamin C intake (OR = 0.39; 95%CI 0.15-1.00; P-trend=0.87). Mean serum concentrations were significantly lower in cases than in controls (P=0.012) and a significant reduction in fracture risk for quartile 4 compared to quartile 1 of serum concentrations was found (OR = 0.31; 95%CI 0.11-0.87; P-trend=0.03).
Park [340] 2011 South Korea	*n* 72 osteoporotics; 72 controls (women)	50-70	FFQ	**Dietary intake:** Quartile 1 ≤ 91.5 mg/d Quartile 2 = 91.5-136.9 mg/d Quartile 3 = 136.9-176.3 mg/d Quartile 4 > 176.3 mg/d	Dietary vitamin C intake & Risk of osteoporosis	Intake: S	A significant reduction in the risk of osteoporosis for quartile 3 compared to quartile 1 of dietary vitamin C intake (OR = 0.29; 95%CI 0.09-0.96; P-trend=0.24)

CA, cases; CO, controls; sign, significant; NS, not significant.

Table A2.4: Cross-sectional studies on vitamin C and bone health.

Study	Subjects	Age (yrs)	Dietary assessment	Mean (SD); range vitamin C intake or blood levels	Outcome measures and analyses	Results	Comments
Sowers [344] 1985 US	*n* 324 (women)	67 (55-80)	24hR	**Total intake:** Low calcium group = 211 (351) mg/d High calcium group = 268 (309) mg/d	Association between MR BMD and vitamin C intake	Total: NS	Vitamin C intake was only marginally associated with MR BMD (*Effect size not shown*; *P*=0.051).
Leveille [341] 1997 US	*n* 1892 (women)	72 (55-64)	FFQ	**Dietary intake** = 113 (52); 12-399 mg/d **Suppl. intake** = 294 (447); 0-2500 mg/d Duration of suppl. use: Group 1 = non-user Group 2 = 1-5 yrs Group 3 =5-10 yrs Group 4 ≥ 10 yrs **Total intake** = 407 (454); 13-2560 mg/d	FN BMD stratified by vitamin C intake or FN BMD stratified by duration of vitamin C suppl. use and either age groups (55-64yrs, 65-74yrs and 75+) or oestrogen use	Diet: NS Suppl: M Total: NS	No significant associations between dietary or total vitamin C intake and FN BMD. Approximately 6.7% and 3.2% higher FN BMD for longest supplement users compared to non-users in women aged 55-64yrs (*P*=0.02; P-trend=0.01) and in women who had never taken oestrogen (*P*=0.02; P-trend=0.02), respectively. No significant differences in older age groups. The duration of supplement use did not affect FN BMD in past oestrogen users and in the combined population sample.
New [133] 1997 UK	*n* 994 (women)	47 (44-50)	FFQ	**Dietary intake** = 126 (96); 16-1164 mg/d *Intake data for quartiles not shown.*	LS, FN, T and WT BMD stratified by quartiles of dietary vitamin C intake	Diet: S	Dietary vitamin C intake correlated significantly with LS BMD (r^2=0.10; *P*<0.001). Approximately 4.5% higher LS BMD (*P*<0.002), 3% higher FN BMD (*P*<0.01) and higher T and WT BMD *(Effect sizes not shown*; *P*<0.02*)* for quartile 3 *vs.* 1 of vitamin C intake.
Hall [91] 1998 US	*n* 775 (women)	56 (45-64)	FFQ	**Dietary intake** = 140 (76) mg/d *Note: dietary calcium intake:* *Low (n 199) < 500 mg/d* *High (n 574) > 500 mg/d*	LS, FN and TH BMD stratified by 100mg/d increments of vitamin C intake with and without stratification by low and high calcium intake	Diet: M	FN and TH BMD were 0.017 g/cm^2 higher for each 100 mg/d increase in vitamin C intake (*P*=0.002 and *P*=0.005). The 0.014 g/cm^2 increment in LS BMD was not significant (*P*=0.078). Moreover, for every 100 mg/d increment in dietary vitamin C intake a significant 0.0199 g/cm^2 increment at the LS (*P*=0.024), 0.0190 g/cm^2 increment at the FN (*P*=0.002) and 0.0172 g/cm^2 increment at the TH (*P*=0.010) was found for the high calcium group. No significant associations in the low calcium group.
New [124] 2000 UK	*n* 62 (women)	47 (45-54)	FFQ	**Dietary intake** = 103 (66); 24-453 mg/d *Intake data for quartiles not shown.*	LS, FN, T, WT and forearm BMD and markers of bone metabolism (PYD, DPD, OC) stratified by quartiles of dietary vitamin C intake	Diet: M	Dietary vitamin C intake did not correlate with markers of bone metabolism. But significantly lower mean DPD excretion across quartiles of dietary vitamin C intake (*Effect size not shown*; *P*-trend<0.02). No significant differences between dietary vitamin C intake and any BMD site.

Table A2.4: (continued)

Study	Subjects	Age (yrs)	Dietary assessment	Mean (SD); range vitamin C intake or blood levels	Outcome measures and analyses	Results	Comments
Morton [165] 2001 US	*n* 994 (women)	72 (50-98)	N/A	**Suppl. intake:** Non-users = 0 mg/d Users = 745 mg/d; 70-5000 mg/d Group 1 = 0 mg/d (non-users) Group 2 ≤ 500 mg/d Group 3 ≥ 1000 mg/d	LS, FN, TH, MR and UR BMD stratified by use of vitamin C suppl. with and without additional stratification by oestrogen use or by oestrogen and calcium use; and BMD stratified by dose of vitamin C supplements	Suppl.: M	4.1% higher FN BMD for supplement users compared to non-users (P=0.02). No significant differences at other BMD sites and in BMD between users and non-users of vitamin C and oestrogen supplements. For current users of oestrogen, calcium and vitamin C supplements, BMD was higher by approximately 6% at the TH (P=0.05), 9% at the FN (P=0.0001) and 12% at the UR (P=0.02) compared to non-vitamin C users. Approximately 14% higher UR BMD for women with the highest vitamin C supplement dose compared to non-users (P<0.05; P-trend<0.04). No significant differences at other bone sites.
Simon [169] 2001 US	*n* 13080 (6137 men; 6943 women)	(20-90)	24hR	**Men:** Dietary intake = 102 (104) mg/d Serum levels = 38.0 (23.8) µmol/L **Pre-menopausal women:** Dietary intake = 81 (83) mg/d Serum levels = 43.7 (25.6) µmol/L **Post-menopausal women:** Dietary intake = 88 (80) mg/d Serum levels = 50.5 (27.8) µmol/L	TH BMD or self-reported fractures stratified by 100 mg/d increments in dietary vitamin C intake or by SD increments in serum ascorbic acid levels	Diet: M Serum: M	In men, TH BMD was highest at serum ascorbic acid concentrations between about 28.4-56.8 µmol/l and self-reported fractures were least common at dietary vitamin C intakes of about 200 mg/d; whereas higher and lower levels were associated with lower TH BMD (P<0.05) and a higher self-reported fracture prevalence (P=0.01). In pre-menopausal women, TH BMD was 0.01 g/cm^2 higher for every 100 mg/d increase in dietary vitamin C intake (P=0.002). No such observations in postmenopausal women. Dietary vitamin C intake or serum ascorbic acid levels were also not significantly associated with self-reported fractures in women.
Ilich [345] 2003 US	*n* 136 (women)	69 (57-88)	3dDD	**Dietary intake** = 128 (70); 23-402 mg/d	Dietary vitamin C intake as a predictor of WB BMD and BMC and of TH, FN, WT, T, RS, UR and hand BMD	Diet: S	Dietary vitamin C intake was a predictor of BMD of more than 1% for TH (P=0.012), T (P=0.047) and RS (P=0.027) BMD and a marginally significant predictor of WT BMD (P=0.052).

Table A2.4: (continued)

Study	Subjects	Age (yrs)	Dietary assessment	Mean (SD); range vitamin C intake or blood levels	Outcome measures and analyses	Results	Comments
Wolf [342] 2005 US	*n* 11068 (women)	63 (50-79)	FFQ	**Dietary intake** = 84 (49) mg/d **Total intake** = 170 (182) mg/d	WB, LS, TH, FN and T BMD stratified by dietary or total vitamin C intake and additional stratification by either calcium intake, smoking or HRT use	Diet: NS Total: NS	No significant associations between dietary or total vitamin C intake and BMD at any site. But there was a significant positive interaction effect between HRT use and total vitamin C intake for WB (*P*=0.045), LS (*P*=0.03), TH (*P*=0.029) and FN (*P*=0.004) BMD. No significant interactions between vitamin C intake and calcium intake or smoking for any BMD site.
Pasco [343] 2006 Australia	*n* 533 (women)	56-82	N/A	*Intake data not shown.* **Duration of suppl. use** (vitamin C + E): Group 1 = 0 yrs (non-user) Group 2 < 5 yrs Group 3 ≥ 5 yrs	WB BMD, serum CTx and BSALP stratified by use or duration of vitamin C and E supplements	Suppl.: M	No significant differences in (unadjusted) WB BMD, CTx and BSALP between users and non-users of vitamin C and E supplements. Duration of vitamin C and E supplement use of ≥5 years was associated with significantly lower CTx levels compared to non-supplement users (*P*<0.05). CTx levels were 0.022 pg/mL lower for each year of vitamin supplement use (*P*=0.05). BSALP and WB BMD were not associated with duration of supplement use.
Prynne [131] 2006 UK	*n* 257 (111 boys; 101 girls); *n* 67 (older women)	17 (16-18); 68 (60-83)	7dDD	**Dietary intake:** Boys = 96 mg/d Girls = 95 mg/d Older women = *Data not shown.*	WB, LS, TH, FN and T BMD stratified by vitamin C intake	Diet: M	In boys, each 100% change vitamin C intake was associated with a 3-5% change in BMD at all sites (*P*<0.05). No significant associations in girls and older women.
Sahni [230] 2008 US	*n* 874 (334 men; 540 women)	75	FFQ	**Dietary intake:** Men = 141 (73) mg/d Women = 158 (83) mg/d **Suppl. intake:** Men = 82 (235) mg/d Women = 95 (248) mg/d **Total intake:** Men = 223 (259) mg/d Women = 253 (267) mg/d *Intake data for tertiles not shown.*	LS, FN, T and RS BMD stratified by tertiles of dietary or total vitamin C intake or categories of suppl. vitamin C intake and either calcium intake, vitamin E intake, smoking or oestrogen use	Diet: NS Suppl.: M Total: M	In men, total vitamin C intake was positively associated with FN BMD but only among never-smokers (*P*-trend=0.04). In current smokers, total and supplemental vitamin C intake were negatively associated with T BMD (*P*-trends=0.01). No significant associations between dietary vitamin C intake and BMD in men and no significant associations in women for any of the BMD sites.

Table A2.4: (continued)

Study	Subjects	Age (yrs)	Dietary assessment	Mean (SD); range vitamin C intake or blood levels	Outcome measures and analyses	Results	Comments
Sugiura [229] 2011 Japan	*n* 293 (women)	60	FFQ	**Dietary intake** = 170 (161-179) mg/d† Tertile 1 = 47-139 mg/d‡ Tertile 2 = 140-214 mg/d Tertile 3 = 215-625 mg/d	Risk of low radial BMD stratified by tertiles of dietary vitamin C intake	Diet: S	Significantly lower risk of low radial BMD for tertile 3 compared to tertile 1of dietary vitamin C intake (OR = 0.25; 95%CI 0.07-0.82; *P*-trend=0.01).

MR, mid radius; NS, not significant; Suppl, supplement; FN, femoral neck; LS, lumbar spine; T, trochanter; WT, Ward's triangle; sign, significant; TH, total hip; PYD, pyridinoline; DPD, deoxypyridinoline; OC, osteocalcin; UR, ultradistal radius; WB, whole body; RS, radial shaft; CTx, collagen type 1 cross-linked C-telopeptide; BSALP, bone-specific alkaline phosphatase.

** Data shown for men / women.*

† Geometric mean (95%CI).

‡ Intake range.

Appendix 3: Associations between total vitamin C intake and fracture risk

Table A3.1: Results in men

Table A3.2: Results in women

Table A3.1: Associations between total vitamin C intake and fracture risk in men of the EPIC-Norfolk case-cohort.

		Total vitamin C intake (mg/d)									
		Quintile 1 **0 – 45.8** ***n = 411***		**Quintile 2** **45.9 – 64.4** ***n = 410***		**Quintile 3** **64.5 – 92.1** ***n = 411***		**Quintile 4** **92.2 – 130.2** ***n = 410***		**Quintile 5** **130.3 – 1595.3** ***n = 410***	
		HR (ref)	**HR**	**(95% CI)**	**HR**	**(95% CI)**	**HR**	**(95% CI)**	**HR**	**(95% CI)**	***P*-trend**
Total fracture	[Events]	[61]		[46]		[53]		[38]		[50]	
	Unadjusted	1.00	0.68	(0.44-1.04)	0.83	(0.55-1.25)	**0.54**	**(0.35-0.85)****	0.78	(0.51-1.18)	*P*=0.14
	Model 1	1.00	0.68	(0.44-1.05)	0.82	(0.54-1.25)	**0.54**	**(0.35-0.85)****	0.79	(0.51-1.20)	*P*=0.17
	Model 2	1.00	0.66	(0.43-1.03)	0.80	(0.52-1.23)	**0.52**	**(0.33-0.83)****	0.76	(0.49-1.18)	*P*=0.15
Hip fracture	[Events]	[28]		[22]		[26]		[18]		[18]	
	Unadjusted	1.00	0.70	(0.38-1.29)	0.88	(0.50-1.58)	0.55	(0.29-1.04)	0.61	(0.32-1.15)	*P*=0.09
	Model 1	1.00	0.68	(0.36-1.27)	0.88	(0.48-1.60)	0.54	(0.28-1.04)	0.60	(0.31-1.15)	*P*=0.09
	Model 2	1.00	0.72	(0.38-1.37)	0.92	(0.49-1.73)	0.55	(0.27-1.10)	0.64	(0.33-1.25)	*P*=0.13
Spinal fracture	[Events]	[19]		[11]		[15]		[13]		[20]	
	Unadjusted	1.00	0.51	(0.24-1.10)	0.73	(0.36-1.48)	0.67	(0.33-1.37)	0.98	(0.51-1.90)	*P*=0.82
	Model 1	1.00	0.51	(0.23-1.13)	0.74	(0.37-1.48)	0.66	(0.32-1.35)	1.00	(0.51-1.97)	*P*=0.80
	Model 2	1.00	0.49	(0.22-1.08)	0.69	(0.34-1.42)	0.63	(0.30-1.33)	0.91	(0.43-1.95)	*P*=0.93
Wrist fracture	[Events]	[17]		[17]		[12]		[8]		[16]	
	Unadjusted	1.00	0.99	(0.49-1.97)	0.70	(0.33-1.48)	0.46	(0.20-1.08)	0.93	(0.46-1.86)	*P*=0.36
	Model 1	1.00	1.00	(0.49-2.02)	0.72	(0.34-1.52)	0.47	(0.20-1.11)	0.97	(0.48-1.98)	*P*=0.45
	Model 2	1.00	0.90	(0.44-1.82)	0.60	(0.27-1.31)	**0.41**	**(0.17-0.99)***	0.86	(0.42-1.76)	*P*=0.29

*Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). Total vitamin C intake is the sum of vitamin C intake from foods and supplements. The analysis used data from the first health check. Significant differences between the two upper quintiles referent to the lowest quintile: * (P<0.05), ** (P<0.01). Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity and use of steroids. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 1957 for total fracture, n 1842 for hip fracture, n 1808 for spine fracture, n 1806 for wrist fracture.*

Table A3.2: Associations between total vitamin C intake and fracture risk in women of the EPIC-Norfolk case-cohort.

		Total vitamin C intake (mg/d)								
		Quintile 1 **0.1 – 50.6** ***n = 592***		**Quintile 2** **50.7 – 73.1** ***n = 592***	**Quintile 3** **73.2 – 99.7** ***n = 592***		**Quintile 4** **99.8 – 141.3** ***n = 592***		**Quintile 5** **141.4 – 6141.9** ***n = 591***	
		HR (ref)	**HR**	**(95% CI)**	**HR**	**(95% CI)**	**HR**	**(95% CI)**	**HR**	**(95% CI)**	***P*-trend**
Total fracture	[Events]	[136]		[126]		[117]		[115]		[122]	
	Unadjusted	1.00	1.04	(0.77-1.40)	0.97	(0.72-1.31)	0.93	(0.69-1.27)	1.07	(0.79-1.45)	*P*=0.92
	Model 1	1.00	1.09	(0.80-1.48)	0.99	(0.72-1.34)	0.96	(0.71-1.31)	1.12	(0.82-1.53)	*P*=0.79
	Model 2	1.00	1.11	(0.81-1.51)	1.01	(0.74-1.37)	0.99	(0.72-1.37)	1.16	(0.83-1.61)	*P*=0.66
Hip fracture	[Events]	[71]		[68]		[61]		[74]		[65]	
	Unadjusted	1.00	1.14	(0.77-1.69)	1.05	(0.70-1.56)	1.28	(0.87-1.88)	1.19	(0.80-1.77)	*P*=0.30
	Model 1	1.00	1.24	(0.82-1.85)	1.08	(0.72-1.62)	1.36	(0.91-2.02)	1.19	(0.79-1.81)	*P*=0.32
	Model 2	1.00	1.28	(0.85-1.92)	1.12	(0.74-1.69)	1.42	(0.95-2.14)	1.25	(0.81-1.95)	*P*=0.24
Spinal fracture	[Events]	[36]		[24]		[19]		[19]		[26]	
	Unadjusted	1.00	0.73	(0.42-1.25)	0.60	(0.33-1.07)	0.58	(0.32-1.02)	0.87	(0.351-1.47)	*P*=0.37
	Model 1	1.00	0.79	(0.45-1.19)	0.65	(0.36-1.19)	0.61	(0.34-1.10)	0.95	(0.54-1.69)	*P*=0.54
	Model 2	1.00	0.80	(0.45-1.40)	0.66	(0.36-1.21)	0.63	(0.34-1.16)	1.01	(0.55-1.87)	*P*=0.68
Wrist fracture	[Events]	[51]		[45]		[44]		[33]		[45]	
	Unadjusted	1.00	0.92	(0.60-1.41)	0.93	(0.61-1.42)	0.65	(0.41-1.03)	0.99	(0.65-1.51)	*P*=0.49
	Model 1	1.00	0.93	(0.60-1.43)	0.89	(0.58-1.37)	0.64	(0.40-1.02)	0.98	(0.64-1.51)	*P*=0.45
	Model 2	1.00	0.94	(0.61-1.45)	0.89	(0.57-1.37)	0.63	(0.39-1.02)	0.94	(0.60-1.49)	*P*=0.37

Values are Prentice-weighted Cox proportional hazard ratios of fracture risk after a median follow-up of 12.6 years (with 95%CIs). Total vitamin C intake is the sum of vitamin C intake from foods and supplements. The analysis used data from the first health check. No significant differences between the two upper quintiles referent to the lowest quintile. Model 1 adjusted for age, family history of osteoporosis, BMI, smoking, physical activity, use of steroids, menopausal status and HRT. Model 2 additionally adjusted for energy intake, dietary calcium intake, calcium supplements and vitamin D supplements. n 2754 for total fracture, n 2525 for hip fracture, n 2334 for spine fracture, n 2409 for wrist fracture.

Appendix 4: Literature tables for chapter 6

(Iron & bone health)

Table A4.1: Intervention studies

Table A4.2: Prospective studies

Table A4.3: Cross-sectional studies

Table A4.1: Summary of intervention studies on iron and bone health.

Study	Subjects	Age (yrs)	Duration; study design	Intervention	Primary outcome	Results*	Comments
Toxqui [180] 2014 Spain	*n* 165 (women)	25 ± 4 (18-35)	4 months; double-blind RCT	3 groups: Control group (*n* 56, iron sufficient): no supplementation; Fe group (*n* 54, iron deficient): iron (15 mg/d) via fortified skimmed milk; Fe+D group (*n* 55, iron deficient): iron (15 mg/d) and vitamin D_3 (5 µg/d) via fortified skimmed milk	PINP, NTx	M	Significant negative correlations between transferrin and PINP (R^2=0.058, *P*=0.002) and between ferritin and NTx (R^2=0.079, *P*<0.001) at baseline in all subjects. The iron-fortified milk did not improve iron status over the 16 weeks. Subsequently, PINP and NTx levels were not affected by iron supplementation (Fe group). However, the addition of vitamin D (Fe+D group) significantly decreased bone marker concentrations from baseline to 16 weeks (PINP: from 53.3 to 48.4, *P*=0.004; NTx: from 64.0 to 47.4, *P*<0.001).
Blanco-Rojo [179] 2013 Spain	*n* 41 (women)	26 ± 6 (18-35)	4 months; double-blind RCT	2 groups (both iron deficient): Control group (*n* 18): 500 ml/d placebo fruit juice; Fe group (*n* 23): 500 ml/d iron-fortified fruit juice (18 mg/d of iron pyrophosphate)	BSALP, NTx	NS	BSALP and NTx concentrations did not change over the four months in neither the iron-fortified group nor the control group.
Wright [178] 2013 Spain	*n* 73 (women)	35 ± 5 and 28 ± 3 (anaemics and controls)	2-4 months; *not reported*	2 groups: Control group (*n* 38, iron sufficient); Anaemic group (*n* 35): iron (80-160 mg/d) via ferrous sulphate tablets. Further split into two subgroups of those that recovered (*n* 22) and did not recover (*n* 13) from iron deficiency	PINP, NTx	M	At baseline, anaemic women had significantly higher NTx levels than controls (37.8±16.5 *vs.* 21.9±8.4 nmol bone collagen equivalents (BCE) / mmol creatinine, *P*<0.001). There were no differences in PINP and NTx at baseline and after iron supplementation between recovered and non-recovered anaemic women. However, both bone markers decreased significantly during the iron supplementation in recovered women only (before *vs.* after treatment: PINP: 41.2±17.5 *vs.* 32.6±14.5 ng/ml, *P*<0.001; NTx: 40.0±17.2 *vs.* 31.0±9.9 nmol BCE / mmol creatinine, *P*<0.05).

Abbreviations: BSALP, bone-specific alkaline phosphatase; NTx, collagen type 1 cross-linked N-telopeptide; PINP, procollagen type I propeptide.
** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table A4.2: Prospective studies on iron and bone health.

Study	Subjects	Age (yrs)	Follow-up	Dietary assessment	Mean ± SD (range) iron intake or blood levels	Outcome measures and analyses	Results*	Comments
Maurer [405] 2005 US	*n* 228 (women)	56 ± 5 (40-65)	1 yr	8 randomly selected days from 2-3 week diet records taken at 0, 6 and 12 months	**Dietary intake:** 15 ± 5 mg/d	Associations between iron intake and 1-year change in TB, LS, FN, T and WT BMD	Diet: M	Iron was significantly positively associated with 1-year change in T BMD (β 0.041, *P*=0.015) and WT BMD (β 0.055, *P*=0.037). No associations with TB, LS and FN BMD. Iron accounted for 3-9% of the variance in BMD change.
Abraham [186] 2006 UK	*n* 32 (women)	NR (46-55)	3.5-5 yrs	7-13 weighed 3dDD or 7dDD	**Dietary intake:** 12.0 ± NR (6.2-24.7) mg/d	Associations between iron intake and 3.5-5 year change in LS BMD	Diet: S	Energy-adjusted iron intake correlated significantly with the preservation of LS BMD over time (r=0.42, *P*=0.02). Higher iron intake was also significantly associated with less LS BMD loss after correcting for energy intake and BMI (β 0.141, *P*<0.0001).
Kim [185] 2012 Korea	*n* 1729 (789 men; 940 women)	56 ± 8 (NR)	3 yrs	N/A	**Serum ferritin:** Men = 147.3 ± 83.6 ng/ml Women = 76.9 ± 50.6 ng/ml	Associations between serum ferritin and annualised 3-year change in FN, T and TH BMD	Status: M	Subjects in quartile 4 compared to 1 had significantly faster annualised bone loss at the FN and TH (women: 34-37%; men: 78-113%; *P*≤0.023); and the trends across all quartiles were also significant (*P*-trend≤0.043).
						Associations between serum ferritin and morphological vertebral fracture risk	Status: M	In women, the odds for fractures were significantly higher in quartile 4 compared to 1 (OR 5.27, 95%CI 1.12-24.94), and this inverse association across all quartiles was significant (*P*-trend=0.023). No such associations were found in men.

TB, total body; LS, lumbar spine; FN, femoral neck; T, trochanter; WT, Ward's triangle; NR, not reported; N/A, not applicable.
** Results were significant (S), non-significant (NS) or of mixed nature (M).*

Table A4.3: Cross-sectional studies on iron and bone health.

Study	Subjects	Age (years)	Dietary assessment	Mean ± SD (range) iron intake or blood levels	Outcome measures and analyses	Results*	Comments
Angus [407] 1988 Australia	*n* 159 (women)	38 ± 8 (pre-mp), 59 ± 8 (post-mp), (23-75)	4dDD (weighed)	**Dietary intake:** Pre-mp = 10.9 ± 3.8 mg/d Post-mp = 9.9 ± 3.4 mg/d	Association between LS, FN, WT, GT BMD and forearm BMC and iron intake	Diet: M	In pre-mp women, there were positive correlations between iron intake and FN BMD (r=0.24) and forearm BMC (r=0.26) (all *P*<0.05). Iron intake was also an independent predictor of FN BMD alongside age and weight (R^2=0.25, *P*<0.001). No associations were found in post-mp women.
Michaëlsson [406] 1995 Sweden	*n* 175 (women)	51 ± NR (28-74)	FFQ, four 7dDDs	**Dietary intake:** FFQ = 11.4 ± 3.9 (3.4-34.9) mg/d 7dDD = 12.3 ± 3.3 (3.6-20.4) mg/d	Associations between TB, LS, FN, WT and T BMD and serum OC with iron intake	Diet FFQ: NS 7dDD: M	Iron intake (7dDD) was significantly and positively associated with all BMD sites but only in univariate analyses (β 0.0069-0.011, *P*≤0.02). No significant associations with serum OC. Iron intake (FFQ) was not associated with BMD and serum OC.
Harris [181] 2003 US	*n* 242 (women)	55 ± 5 (40-66)	3dDD	**Dietary intake:** 16 ± 6 mg	Associations between TB, LS, FN, WT and T BMD with iron intake	Diet: S	Iron intake was significantly and positively associated with adjusted BMD at all sites (β 0.085-0.251, *P*≤0.01). In linear regression analyses, BMD at all sites increased by 4-14% between extreme quartiles of iron intake (*P*≤0.05).
				Categories of iron intake: Cat.1 = <10 mg Cat.2 = 10-14 mg Cat.3 = 14-20 mg Cat.4 = 20-40 mg	Interaction effects between iron and calcium intake on TB, LS, FN, WT and T BMD		For women with calcium intakes of 800-1200 mg/d, those that had iron intakes of ≥20 mg/d had higher BMD at all sites than those with iron intakes of <10 mg/d *(results visualised in graph, no effect sizes and significance levels reported)*.

Table A4.3: *(continued)*

Study	Subjects	Age (years)	Dietary assessment	Mean ± SD (range) iron intake or blood levels	Outcome measures and analyses	Results	Comments
Cesari [184] 2005 Italy	*n* 950 (420 men, 530 women)	75 ± 7 (65-102)	N/A	**Anaemia, defined as:** Men: Hb levels < 13 g/dl Women: Hb levels < 12 g/dl	Differences in TRAB and CORT bone density between subjects with and without anaemia	Anaemia: M	In unadjusted analyses, women with anaemia had significantly lower bone density at all sites than those without anaemia (differences = 20.4 – 75.8 mg/cm^3, *P*≤0.02). In men, anaemia subjects had significantly lower cortical bone density only (difference = 26.4 mg/cm^3, *P*=0.01).
					Associations between anaemia and Hb levels with TRAB and CORT bone density		In adjusted analyses, every SD increase in bone density (at different sites) was significantly associated with higher haemoglobin levels in both sexes (β 0.076-0.112, *P*≤0.04) and with a lower prevalence of anaemia in women only (β -0.335-(-)0.428, *P*≤0.04).
					The relationship between Hb levels above and below anaemia cut-off points with TRAB and CORT bone density		In adjusted analyses, women with Hb levels above the anaemia cut-off had significantly higher bone density at multiple sites than those with anaemia (differences = 26.9-39.4 mg/cm^3, *P*≤0.03). In men this was only significant for cortical bone density (difference = 34 mg/cm^3, *P*=0.01).
D'Amelio [183] 2008 Italy	*n* 455 (women)	66 ± 10 (NR)	N/A	**Serum iron:** 92-99 ± 22-26 µg/dl [+] Serum transferrin: 236-267 ± 33-37 mg/dl [+] Serum ferritin: 74-85 ± 31-67 ng/dl [+]	Correlations between serum iron, transferrin and ferritin with LS and FN BMD	Status: M	Serum transferrin significantly correlated with BMD at both sites (LS: R=-0.2, *P*=0.015; FN: R=-0.34, *P*<0.001). No other correlations were reported.
Farrell [142] 2009 US	*n* 244 (women)	56 ± 5 (NR)	FFQ and eight 24hRs	**Dietary intake:** FFQ = 14 ± 6 mg/d 24hR = 15 ± 5 mg/d	The agreement between multiple linear regression of each intake method with TB, LS, FN, T and WT BMD	Diet: S	Iron intake was significantly associated with adjusted BMD at all sites regardless of the dietary assessment used (24hR: β 0.214-0.380 g/cm^3; FFQ: β 0.232-0.426 g/cm^3).

Table A4.3: *(continued)*

Study	Subjects	Age (years)	Dietary assessment	Mean ± SD (range) iron intake or blood levels	Outcome measures and analyses	Results	Comments
Kim [409] 2013 Korea	*n* 5148 (2621 men; 2527 women)	Men: 45 ± 19 (10-93); Women: 44 ± 18 (10-95)	N/A	**Serum ferritin in people aged ≥45 years:** Men = 118.9 (95%CI 115.0-122.9) ng/ml Women = 58.1 (95%CI 55.8-60.4) ng/ml	Associations between serum ferritin levels and LS, FN and TH BMD stratified by age and sex	Status: M	In men, serum ferritin levels were not associated with BMD at any site in any age group. In women, ferritin was significantly inversely associated with all BMD sites in women aged ≥45 years and with LS BMD only in 25-44 year old women (β -0.012-(-)0.039 ± 0.005-0.007 (SE), *P*≤0.041).
				Serum ferritin in women aged ≥45 years: Quartile 1 = 1.1-28.0 ng/ml Quartile 2 = 28.1-50.5 ng/ml Quartile 3 = 50.6-77.9 ng/ml Quartile 4 = 78.0-486.1 ng/ml	Associations between quartiles of serum ferritin levels and LS, FN and TH BMD in women aged ≥45 years	Status: M	Women in quartiles 3 and 4 of ferritin levels had significantly lower LS BMD (approximately 3.2% and 3.4%, respectively) compared to those in quartile 1 (*P*<0.05), and this inverse association was linear (*P*-trend<0.001). No such associations were found for FN and TH BMD.
					Associations between quartiles of serum ferritin levels and the odds for prevalent osteoporosis and self-reported fractures in women aged ≥50 years	Status: S	The odds for osteoporosis were significantly higher in women in quartiles 3 (OR 1.45, 1.02-2.05) and 4 (OR 1.55, 1.09-2.23) compared to quartile 1 of serum ferritin, and this association was linear (*P*-trend=0.013). The odds for self-reported fractures was also significantly higher in women in quartile 4 *vs.* 1 (OR 1.52, 1.02-2.27), with a significant linear trend across all quartiles (*P*=0.034).
Lee [182] 2013 South Korea	*n* 2943 (1371 men; 1569 women)	72 ± 11 (NR)	N/A	**Serum ferritin:** Men = 127.7 ± 6.2(SE) ng/ml Women = 77.1 ± 3.1(SE) ng/ml	Associations between serum ferritin levels and LS, FN and TH BMD	Status: M	Serum ferritin levels were significantly and positively associated with all BMD sites in men (β 0.008-0.018 ± 0.004-0.005 (SE), *P*≤0.049). Moreover, with increasing tertiles of serum ferritin, BMD at all sites increased and the prevalence of osteoporosis decreased significantly in men (*P*≤0.022). No such observations were found in women.

Table A4.3: *(continued)*

Study	Subjects	Age (years)	Dietary assessment	Mean ± SD (range) iron intake or blood levels	Outcome measures and analyses	Results	Comments
Okyay [408] 2013 Turkey	*n* 728 (women)	57 ± 6 (47-79)	N/A	**Serum iron in 45-59 yr age group (*n*=576):** NOP = 101.0-101.3 ± 43.5-45.0 μg/dl ++ OP = 86.8-93.0 ± 43.1-48.1 μg/dl ++ **Serum iron in 60-79 yr age group (*n*=152):** NOP = 106.0-108.7 ± 46.9-50.4 μg/dl ++ OP = 99.6-101.0 ± 46.8-49.7 μg/dl ++	Differences in serum iron levels between those with and without osteoporosis at the LS, FN or TH stratified by age	Status: M	In the younger age group, women with FN and TH OP but not LS OP had significantly lower serum iron levels than NOP women (FN: 101.1±45.0 *vs.* 90.7±43.1 μg/dl, *P*=0.030; TH: 101.0±44.7 *vs.* 86.8±43.4 μg/dl, *P*=0.012). Serum iron did not differ between OP and NOP subjects in the older age group.
					The risk of OP at the LS, FN and TH for those with low *vs.* normal serum iron levels	Status: NS	The risk for OP did not differ between women with low or normal levels of serum iron at any bone site (OR 1.0, 95%CI 0.6-1.9, *P*≥0.33).

Abbreviations: pre-mp, pre-menopausal; post-mp, post-menopausal; LS, lumbar spine; FN, femoral neck; WT, Ward's triangle; GT, greater trochanter, NR, not reported; TB, total body; T, trochanter; OC, osteocalcin; Hb, Haemoglobin; TRAB, trabecular; CORT, cortical; TH, total hip; NOP, no osteoporosis; OP, osteoporosis.

** Results were significant (S), non-significant (NS) or of mixed nature (M).*

+ Values were reported separately for osteoporotic never-fractured women, osteoporotic fractured women and non-osteoporotic women.

++ Values were reported separately for specific bone sites (lumbar spine, total femur and femoral neck).