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CADMIUM AS A RISK FACTOR FOR OSTEOPOROSIS AND FRACTURES IN WOMEN

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To my family

LIST OF PUBLICATIONS

This thesis is based on the following publications, which will be referred to in the text by their Roman numerals.

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LIST OF ABBREVIATIONS

25(OH)D	25-hydroxyvitamin D, Calcidiol
$1,25(OH)_2D$	1,25-dihydroxyvitamin D, Calcitriol
ANCOVA	Analysis of covariance
bALP	Bone alkaline phosphatase
BMC	Bone mineral content (g)
BMD	Bone mineral density (g/cm^2)
BMI	Body mass index (kg/cm^2)
BONETOX	Bone development and homeostasis –critical targets in toxicology
CI	Confidence interval
Cr	Creatinine
CV%	Coefficient of variation %
DXA	Dual energy X-ray absorptiometry
EIA	Enzyme immunoassay
EFSA	European food and safety authority
FFQ	Food frequency questionnaire
GFR	Glomerular filtration rate
g	Gram
HPLC	High pressure liquid chromatography
HRT	Hormone replacement therapy
IARC	International agency for research on cancer
ICD-10	International classification of disease, tenth revision
ICPMS	Inductively chromatography plasma mass spectrometry
IU	International unit
JECFA	Joint FAO/WHO expert committee on food additives
LOD	Limit of detection
MT	Metallothionein
µg	Microgram
NAG	*N*-acetyl-β-D-glucosaminidase
nm	Nanometer
OR	Odds ratio
PHIME	Public health impact of long-term, low-level mixed element exposure in susceptible population strata
Protein HC	Human complex forming glycoprotein (also called α_1-microglobulin)
PTH	Parathyroid hormone
PTWI	Provisional tolerable weekly intake
r_p	Pearson correlation coefficient
r_s	Spearman rank correlation coefficient
SD	Standard deviation
SMC	The Swedish Mammography Cohort
TWI	Tolerable weekly intake
U-Cd	Urinary cadmium
WHILA	The Women's Health in the Lund Area
WHO	World health organization

Units:

1.0 µg cadmium = 8.89 nmol cadmium

1.0 nmol cadmium = 0.11 µg cadmium

1.0 µg cadmium/g creatinine ≈ 1.0 nmol cadmium/mmol cadmium

1.0 mg/dL creatinine = 88.4 µmol/L creatinine

1.0 µmol/L creatinine ≈ 0.011 mg/dL creatinine

1.0 pmol/L $1,25(OH)_2D$ ≈ 0.385 pg/mL $1,25(OH)_2D$

1.0 pg/mL $1,25(OH)_2D$ = 2.6 pmol/L $1,25(OH)_2D$

1.0 nmol/L 25(OH)D ≈ 0.401 nmol/L 25(OH)D

1.0 ng/mL 25(OH)D ≈ 2.50 nmol/L 25(OH)D

1.0 µmol/L serum retinol ≈ 28.7 µg/dL serum retinol

1.0 µg/dL serum retinol = 0.0349 µmol/L serum retinol

ABSTRACT

Cadmium is toxic and accumulates in the body, particularly in the kidneys. Cereals, vegetables and potatoes are the main sources of exposure, besides tobacco smoking. The critical effect of cadmium is considered to be renal damage. Massive exposure is known to cause osteomalacia and osteoporosis with multiple fractures. A few recent studies have indicated that the exposure in the general population is associated with osteoporosis, but the link is not clear.

The aim of this thesis was to investigate effects of long-term low-level cadmium exposure on bone health, and to explore whether these effects were mediated *via* reduced activation of $1,25(OH)_2D$ (vitamin D) in the kidney. Another aim was to elucidate possible combined effects of cadmium and vitamin A (retinol) on bone health. Two population-based studies were used consisting of postmenopausal women, 54 to 69 years of age, with low cadmium exposure. Cadmium exposure was assessed by measuring cadmium concentrations in urine (as a biomarker of long-term exposure) and by estimating the dietary cadmium intake *via* a food frequency questionnaire. Total-body bone mineral density (BMD) and data on fracture incidence (1997-2009) were ascertained. Circulating levels of $1,25(OH)_2D$ and retinol were measured in serum.

Multivariable-adjusted inverse associations were observed between both urinary and dietary cadmium and BMD at the total body, femoral neck, total hip and lumbar spine. We also observed a statistically significant 2-3 fold increased risk of osteoporosis (T-score <-2.5) per µg/g creatinine of urinary cadmium, or per 10 µg/day of dietary cadmium. Among never-smokers, a several-fold statistically significant increased risk of any first fracture, first osteoporotic fracture and first distal forearm fracture was observed for urinary cadmium. A 30-50% statistically significantly increased risk of any first fracture were observed comparing high dietary cadmium intake (≥13 µg/day, median) with lower intakes (<13 µg/day) among all women and never-smokers, respectively. Combined high dietary and high urinary cadmium (≥0.50 µg/g creatinine) as compared to low, a 3-fold statistically significantly increased risk of osteoporosis and fractures were observed among never-smokers.

Urinary cadmium was not associated with $1,25(OH)_2D$ and there was no association between $1,25(OH)_2D$ and markers of bone or kidney effects. This indicates that the negative association between low cadmium levels and BMD was not mediated *via* decreased circulating levels of active vitamin D. Serum retinol concentrations within the normal range tended to be associated with higher BMD at the distal forearm. Serum retinol concentrations in the upper normal range may counteract the negative effect of cadmium on bone.

Altogether this thesis provides important evidence that cadmium exposure at the low exposure levels found in the Swedish general population is associated with negative effects on bone, as indicated by decreased BMD and an increased risk of osteoporosis and fractures. The findings are of high public health relevance since the main dietary cadmium exposure is *via* our most important foods, there are no signs of decreasing exposure levels, and that osteoporosis and related fractures are prevalent.

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1 INTRODUCTION

This thesis focuses on the effects of long-term low level cadmium exposure on bone mineral density (BMD) and on risk of osteoporosis and fractures in women. Osteoporosis and osteoporotic fractures are important and escalating public health problems. The high incidence and the extensive geographical variation (**Figure 1**) [1] in incidence cannot be fully explained by the established risk factors [1-4]. In that context, environmental pollutants have not received much consideration. Cadmium is a widespread environmental pollutant that may exert a wide range of adverse effects on human health, mainly on kidney and bone [5]. The main source of exposure in the general population is food [6, 7], while smokers are additionally exposed. In general, women have higher cadmium body burden than men [6, 8], and are also at higher risk of osteoporosis and related fractures. Whether cadmium should be considered as a risk factor for osteoporosis and fractures remains to be elucidated.

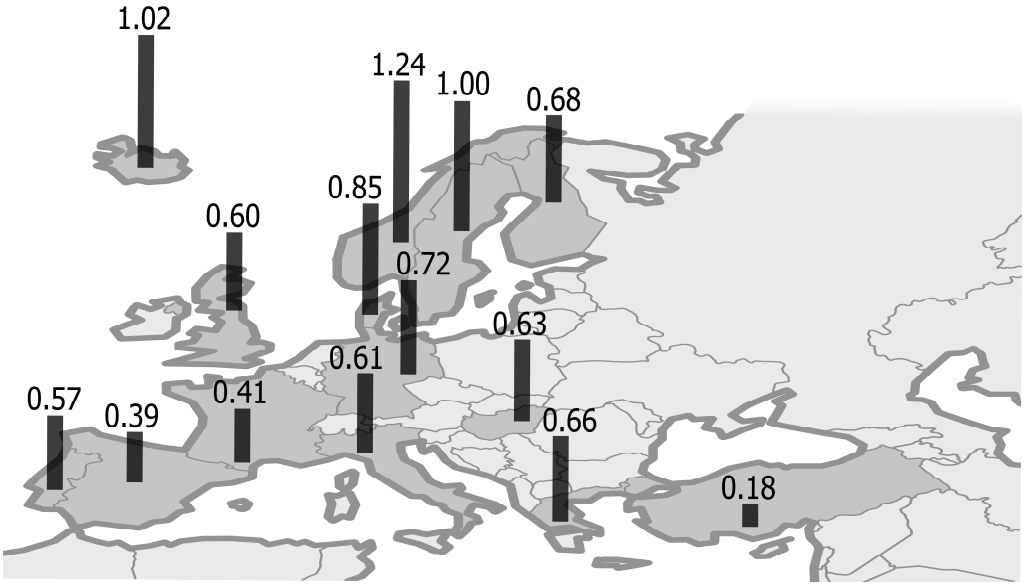

Figure 1. The 10-year probability of hip fractures in men and women adjusted to the probability in Sweden [1].

2 BACKGROUND

2.1 BONE

The skeleton comprises of over 200 bones, each that is first modeled (sculpted) and then remodeled. Depending on the location, the bone supports one or more functions including movement and structural support, and also protects vital organs. The skeleton is the main reservoir for calcium (about 99% is deposited here including in the teeth) and other minerals and is therefore central in the mineral homeostasis [9]. Remodeling constitutes of a delicate balance of bone resorption (osteoclasts) and bone formation (osteoblasts). Remodeling is important for maintenance of the shape of the bone during growth, to repair injuries and is involved in the regulation of serum calcium levels. Osteoblasts produce collagen type 1 (90-95% of the bone matrix consist of collagen type 1), osteocalcin (the most abundant non-collagenous protein) and alkaline phosphatase (an organic phosphate-splitting enzyme). Bone mainly consists of hydroxyapatite [$Ca_{10}(PO_4)_6(OH)_2$], which, in turn, comprises 50% of the adult bone mass. The hydroxyapatite makes the bone hard while the combination of collagen and mineral salts make the bone strong and elastic. The bone mass and calcium metabolism are influenced by several factors such as parathyroid hormone (PTH), $1,25(OH)_2D$ (see below), oestrogen, calcitonin, and different growth hormones [10].

Bone constitutes of two different tissues; the cortical bone (dense or compact) and the trabecular (cancellous) bone, which differ both morphologically and functionally. About 80% of the total bone mass constitutes of cortical bone and the remaining 20% constitutes of trabecular bone. However, the trabecular bone has a larger surface area than the cortical bone. The cortical bone is mainly found in the long bones, while the trabecular is mainly found in the vertebraes (spine), the pelvis and at the ends of the long bones. Thus, the relative proportions vary considerably among the different skeletal sites. For example is the ratio trabecular:cortical estimated to be 75:25 in the vertebraes, and 25:75 in femoral neck and distal forearm. The trabecular bone is more sensitive to hormonal influences and is considered to have a faster rate of metabolism [10].

2.1.1 Measurements of bone mineral density

There are several different methods available to measure BMD but the most common one is dual energy X-ray absorptiometry (DXA); which is also regarded as the reference standard [11, 12]. BMD is expressed as grams of mineral per area of volume (g/cm^2) and can be measured at

total-body and at specific sites such as the distal forearm, femoral neck, hip or spine. When measuring BMD, mainly the calcium (i.e. the amount of mineral) in the bone is measured.

By comparing BMD to a reference population, *T*-score and *Z*-score can be calculated. *T*-score is used to compare the obtained value to the mean value in young adults of the same sex and is calculated as $(BMD_o - BMD_x)/SD$ where BMD_o is the obtained BMD; BMD_x the measured BMD and SD (standard deviation) in the reference population. *T*-score is most often used in clinical practice. If the purpose is to compare the obtained BMD results with a population with of the same age and sex, *Z*-score is used. A common definition of osteoporosis is T-score <-2.5; i.e. 2.5 SD below the mean values of a young adult reference population [13].

BMD may predict the risk of fractures. A meta-analysis showed that BMD at all measured sites had approximately the same predictive abilities [14], i.e. 1 SD decrease in BMD resulted in a relative risk (RR) of a fracture of 1.5 (95% CI, 1.4-1.6). However, the prediction of fracture risk was higher when BMD was measured at the specific site of interest, i.e. measurements of the BMD at the lumbar spine gave a RR of 2.3 (95% CI, 1.9-2.8) for a fracture at the lumbar spine, and BMD measurements at the hip a RR of 2.6 (95% CI, 2.0-3.5) for a fracture at the hip. Thus, the predictive ability of 1 SD decrease in BMD for a fracture is similar or better than that of 1 SD increase in blood pressure for stroke or 1SD increase in serum cholesterol for cardiovascular disease [14].

2.1.2 Osteoporosis and fractures

Osteoporosis is “A systemic skeletal disease, characterized by low bone mass and micro-architectural deterioration of bone tissue with consequent increase in bone fragility and increased fracture risk” as defined by the World health organization [13] (**Figure 2**). The risk of osteoporosis relies on the measurement of BMD but the clinical significance lies in the fractures that arise [11].

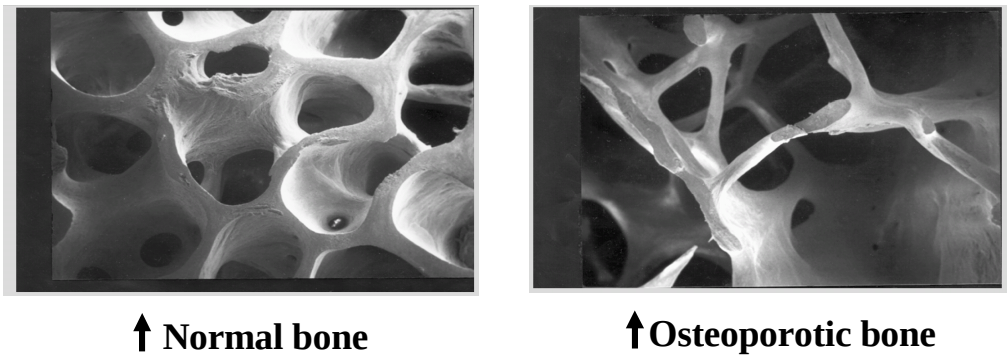

Figure 2. Normal and osteoporotic bone. With permission from the American Society and Bone Mineral Research (ASBMR) (1986, Wiley); Dempster and colleagues [15].

Peak bone mass is achieved in the late second decade or third decade. In middle-aged and in the elderly the resorption of bone exceeds the formation, leading to loss of bone mass. Osteoporosis is reflecting an imbalance of bone formation and bone resorption, in which bone resorption dominates. The disease is silent – there are no symptoms – until the first fracture occurs. The most common osteoporotic fractures are those at the hip, spine, distal forearm, proximal humerus and pelvis [16]. In terms of disability, hip - and vertebral fractures are of particular concern [11]. The mortality the first year after a hip fracture is 10-15% [17].

The incidence of fractures has increased substantially since the 1950ies and is expected to reach 4,5 million by the year 2050 in Europe [2]. Although a large part of this increase is related to an increased ageing population, there is an exceptionally high age-adjusted incidence of osteoporotic fractures in certain Western populations [1, 3] such as Sweden and Norway (**Figure 1**), which together with the observed increased incidence over time worldwide, underline the need to explore all possible risk factors to enable prevention. In Sweden, every other woman and one in four men are statistically expected to have an osteoporotic fracture during their lifetime [17, 18]. Every year 70,000 osteoporotic fractures occur in Sweden; out of these, about 18,000 are hip fractures and 25,000 are fractures at the distal forearm [17]. In Europe, 620,000 hip fractures, 574,000 distal forearm fractures, 250,000 proximal humerus fractures and 620,000 clinical spine fractures occur per year, accounting for 35% of worldwide fractures [11]. This is of course a major public health concern due to the pain, reduced quality of life and life expectancy and the high public health costs associated with the disease [19]. The medical cost in Sweden is estimated to 5,6 billion SEK/year (≅1.1% of the total medical cost) [16]. In total, including loss of quality-adjusted life year (QALY), the estimated cost is 15

billion SEK/year, which is more than the separate medical cost for diabetes or coronary heart disease [16].

Genetic factors play a large role for the peak bone mass (about 70%), leaving about 30% or more for others factors including environmental factors. Low BMD is the main risk factor for fractures, but it alone cannot fully explain all osteoporotic fractures. Other known or proposed risk factors for osteoporosis and fractures are for example, older age, female sex, previous low-energy fracture, smoking, use of glucocorticoids, rheumatoid arthritis, consumption of alcohol, low BMI, low physical activity, liver- and kidney diseases, malabsorption, early menopause, low intake of vitamin D and calcium, and high and low intake of vitamin A [20, 21]. In this context, environmental pollutants such as cadmium including its possible interactions with nutrients, have not received much consideration.

The FRAX®-tool has been developed by the WHO to estimate the 10-year probability of fracture risk in patients, http://www.shef.ac.uk/FRAX/. The factors considered by FRAX® are BMD at the femoral neck, age, female gender, weight, height, previous low-energy fracture, parental history of hip fractures, current smoking, use of glucocorticoids, presence of rheumatoid arthritis, and consumption of alcohol.

2.1.3 Vitamin D

Vitamin D belongs to the family of fat-soluble vitamins and was, when discovered in 1922 primarily considered to be associated with bone health. Vitamin D (25(OH)D) is unique, since it can be obtained from both endogenous production of vitamin D_3 (in the skin), and exogenous sources (mainly D_3) from foods such as fatty-fish, fish liver oils, liver, egg yolks and cheese. Vitamin D is also present in fortified foods such as low-fat dairy products and in dietary supplements. The recommended daily intake in Sweden is 7.5-10 µg/day, but the estimated average intake is much lower [22, 23]. Vitamin D status is reflected by the concentrations of 25(OH)D in serum. During the winter, at Northern latitudes above 40° (Sweden is at latitude 55-69°), the cutaneous synthesis of pre-vitamin D_3 is not detectable [24] and therefore dietary sources such as fatty fish and fortified foods (reduced-fat dairy products and margarines), are of great importance during these months [22].

Vitamin D coming from the skin or diet enters the circulation and is first metabolized in the liver and then converted in the kidney to $1,25(OH)_2D$ - the bioactive form of vitamin D [10].

The role of vitamin D in calcium and phosphate absorption and metabolism is well-known, and thus also its importance for bone health. The physiological effect of $1,25(OH)_2D$ is to maintain plasma concentrations of calcium and phosphate and also to stimulate osteoblasts to synthesize osteocalcin. $1,25(OH)_2D$ primary target tissue is the small intestine, however is also acts on the kidney and bone [25, 26].

2.1.4 Vitamin A

Vitamin A also belongs to the group of fat-soluble vitamins; with the two main groups being retinoids or pro-vitamin A. Retinoids include retinol and its metabolites – with retinoic acid having the biological activity, exerting its activity *via* binding to and activation of the retinoic acid receptor (RAR). Retinoic acid can also bind to the retinoic X receptor (RXR). With regard to pro-vitamin A (e.g. β-carotene), only 10% exert vitamin A activity after first being conversed to retinol. In general, vitamin A is important for proliferation and differentiation of cells; and is likely to have a role in bone tissue remodelling, since both osteoblasts and osteoclasts express RARs.

Vitamin A is obtained from food of animal origin, such as liver and dairy products, and from fortified foods, dietary supplements and as pro-vitamin from plant-derived foods [27]. The Nordic nutrition recommendation of vitamin A intake is 900 µg retinol equivalents /day for men and 800 µg for women [28]. The intake of vitamin A (retinol) from food in Sweden, is generally somewhat higher than that recommended [23], mainly due to the fortification of low-fat dairy products and margarine. In addition to the dietary intake of vitamin A, dietary supplements often contain retinol, and it is estimated that, every third woman and every fifth man in Sweden use supplements [29]. Due to recent studies from e.g. Melhus and colleagues; Feskanich and colleagues, and Michaëlsson and colleagues [30-32], indicating a higher risk of fractures with higher vitamin A intake or higher serum retinol concentrations, the level of fortification and the concentration in supplements have been lowered (*www.slv.se*). On the other hand, normal bone metabolism can only be obtained at adequate vitamin A status [33], as also vitamin A deficiency is proposed as risk factor for low BMD and fractures [34, 35].

2.2 CADMIUM

Cadmium is a toxic metal that has no known biological function in the human body. Cadmium was discovered in 1817 as an impurity with zinc carbonate, but it was not until the beginning of the 20th century that cadmium was more used in the industry. Cadmium has been used as a color

pigment (red, yellow, and orange), as stabilizer (e.g. in PVC), cathode in nickel-cadmium batteries, and as coating and platings in order to protect other metals or alloys from corrosion. The main use of cadmium worldwide in 2005 (82%) was in nickel-cadmium batteries [36]. In Sweden, the growing awareness of the adverse health effects of cadmium led in 1982 to a ban of use of certain products with the intention to decrease cadmium concentrations in the environment. Cadmium is released into the environment due to fossil fuel combustion (e.g. coal-fired power plants) where cadmium can be transported long distances from the emission, application of sewage-sludge or phosphate fertilizers to farm land, waste incineration and disposal, and industrial activity including mining and smelting. Cadmium is easily taken up by plants; this uptake is influenced by a number of factors such as pH, competition with other metals and presence of inorganic and organic ligands [6]. Cadmium levels have increased in Swedish soil during the last century [37] but in spite of restricted use of cadmium in several applications since 1982, no decreasing temporal trend in cadmium exposure has been detected [6, 37].

2.2.1 Exposure

In the general non-smoking population, food is the main source of cadmium exposure. High concentrations are present in seafood - such as molluscs, crustaceans, and cephalopods - in offals, in oil seeds and cocoa beans [6]. The major contributing foods (80%) are, however, cereals - especially whole-grains - vegetables and potatoes [6, 38, 39]; i.e. foods generally considered to be healthy which we are recommended to eat more of (*www.slv.se*). The dietary cadmium intake is therefore likely to be higher in certain sub-populations such as vegetarians [40]. In areas with cadmium-contaminated soils, house dust may potentially be another important route of exposure [41]. Smokers are additionally exposed as a result of the high cadmium content of tobacco leaves and the relative high absorption of cadmium in the lungs [6, 42].

2.2.2 Uptake and distribution

Less than 5% of the ingested cadmium is generally considered to be absorbed in the intestine [6, 7], but the absorption may be several-fold higher at iron deficiency due a common transport mechanism of cadmium and iron in the intestine [39, 40, 43-46]. A diet high in fiber, especially cereal fiber, which also contains more cadmium than refined wheat flour, might reduce the cadmium bioavailability as compared to a low-fiber diet (Berglund et al, 1994). The uptake in the lungs is much higher; between 10-50% [6, 42]. After the uptake, cadmium may be bound to

albumin in the blood and transported to the liver [6, 42]. Once cadmium is in the liver it induces metallothionein (MT) – a low molecular weight cysteine-rich protein – which is important for detoxification [42]. MT can also bind essential metals such as copper and. Besides MT, cadmium can also bind to amino acids or to sulhydryl-rich low molecular weight peptides [47]. Since cadmium-MT is a small complex it can be efficiently filtered through the glomerulus and then be reabsorbed in the tubular cells [6, 42]. Cadmium is eliminated very slowly from the body and the half-time in the kidney is 10-30 years [42]. Thus, the cadmium concentration in the kidney increases with age and may peak around 50->60 years, after which it may start to decline [42].

2.2.3 Biomarkers of exposure

The most feasible and commonly used markers of exposure are cadmium in blood and urine. Cadmium in blood, considered the most valid marker of recent exposure is mainly bound to erythrocytes and measured in whole blood or in erythrocytes [5]. Two compartments are suggested to exist; one is related to the recent exposure and has a half-time of 1-3 months, and the other is related to body burden and has a half-time of approximately 10 years [48]. Thus, after long-term low level exposure, cadmium in blood may serve as a good marker of cadmium body burden [5].

Urinary cadmium, on the other hand, is considered to mainly reflect the kidney accumulation [49] (i.e. before onset of tubular proteinuria) and thus the long-term exposure from all sources [6, 42]. Ideally, cadmium should be measured in 24-h urine. This is, however, usually not feasible in large epidemiological studies for practical reasons, and there in an apparent risk of incomplete sampling. Collection of spot urine is more convenient but the dilution of the urine may vary considerably both with regard to water and solutes, within and between individuals, due to variation in fluid intake, temperature, and physical activity [42]. In order to account for this variation the spot urine samples need to be adjusted either for urinary creatinine or for specific gravity.

2.2.4 Kidney effects

Cadmium exposure has been associated with several different health effects such as kidney, bone, and cardiovascular effects and cancer [5]. The critical organ for chronic cadmium exposure has long been considered to be the kidney and renal dysfunction the critical effect [6, 7]. Cadmium induces renal tubular damage, characterized by an increased excretion of low-

molecular weight proteins, such as β-2-microglobulin (β-2-M), α1-microglobulin (also called protein HC) and retinol-binding protein (RBP) and increased excretion of the lysosomal enzyme *N*-acetyl-β-D-glucosaminidase (NAG). If the cadmium exposure continues, tubular damage may progress and also glomerular damage may emerge, with a decreased glomerular filtration rate (GFR). This has been demonstrated in heavily exposed subjects [50-52], but also at much lower exposure levels [53].

During recent years, there has been a debate concerning whether the association observed between urinary cadmium concentrations and urine-based biomarkers of tubular dysfunction at very low exposure levels actually show a causal relationship. If causal, the public health significances of these associations need further evaluation [37], as it has been difficult to ascertain the exact lowest dose for what could be considered a clear adverse effect.

2.2.5 Bone effects

Cadmium's adverse effect on bone became obvious when the Itai-itai disease (Ouch-Ouch) was discovered in the Toyama Prefecture in Japan, more than 50 years ago. This painful disease mainly affected women over 40 years of age that had lived in the area for more than 30 years. The consumption of heavily contaminated rice resulted in severe renal and bone damages followed by multiple fractures [5]. The effects on bone were a combination of osteomalacia and osteoporosis. In 1968, the Japanese Ministry and of Health and Welfare concluded that "Itai-itai disease is caused by chronic cadmium poisoning, on conditions of existence of such inducing factors as pregnancy, lactation, imbalance in internal secretion, aging, and deficiency of calcium" [54]. Approximately 200 subjects were affected and their urinary cadmium concentrations were very high ($\approx$ 30 nmol/mmol creatinine).

First in 1999, the association between cadmium exposure and osteoporosis was examined again [55]. In a prolongation of the ground-breaking CadmiBel study [56] with baseline data (1985-1989) on cadmium in urine, soil and garden vegetables, associations were assessed with BMD at the forearm (SXA) and with incidence of fractures and height loss in 506 women and men. Participants were from cadmium-contaminated areas close to zinc smelters and in control areas in Belgium. Cadmium in urine, leek and soil was inversely associated with BMD at the forearm in postmenopausal women, but not in men. For a doubling of urinary cadmium, an increased risk of fractures at the forearm in women was observed, relative risk (RR) of 1.73 (95% CI,

1.16-2.57) (p=0.007), and height loss in men, RR, 1.60 (95% CI, 0.94-2.72) (p=0.08) [55]. Noteworthy, if age was forced into the multivariable-adjusted model, the increase in RR for urinary cadmium was no longer statistically significant (p=0.08). In both women and men, soil, leek and celery were associated with a similar increased risk of fractures ($p \leq 0.039$) as for urinary cadmium. For height loss, cadmium in leek was associated with an increased risk in both women and men ($p \leq 0.005$), while cadmium in soil and celery were only associated with increased risk of height loss in women ($p \leq 0.035$).

The association between cadmium exposure and bone was confirmed in a Swedish population (n=1021; women and men) living in the proximity of a nickel-cadmium battery plant. Alfvén and colleagues found an increased risk of osteopenia (*Z*-score <-1) and fractures at the distal forearm at urinary cadmium concentrations between 0.50-3 µg/g creatinine, and 2-4 µg/g creatinine, respectively [57, 58]. Some of the participants were occupationally exposed at the battery plant. In studies from heavily polluted areas in China, associations were also observed between cadmium exposure and osteoporosis [59, 60]. When this thesis was initiated only a limited number of studies were available and only one study had examined the association between cadmium and bone at the low exposure levels present in the general "non-exposed" population [61]. In upper middle-aged women in Southern Sweden (Women's Health in the Lund area, WHILA) and inverse association was observed between cadmium in urine, but not in blood, and BMD at the distal forearm; the median urinary cadmium concentration was 0.67 µg/g creatinine. In 2008, Schutte and colleagues found similar associations in a population with environmental cadmium exposure mainly from zinc smelters in Belgium [62]. The studies from Schutte and colleagues, and Åkesson and colleagues suggest a direct effect of cadmium on bone, with increased concentration of bone resorption markers with increasing cadmium exposure, possibly intensified after menopause. Exposure to cadmium was also associated with calciuria and with reactive changes in calciotropic hormones [62]. Because cadmium was associated with lower levels of PTH in both studies, the cadmium-associated calciuria was most likely a result of increased bone resorption, rather than decreased tubular reabsorption followed by an increase in parathyroid hormone [62].

Only one previous study comprising 2,826 women, aged 50 to 90 years, have examined the association between cadmium and BMD outside the site of the distal forearm [63]. An inverse association was observed between urinary cadmium and BMD at the femoral neck and total hip,

and an increased risk of osteoporosis at the total hip [63]. This study (Gallagher et al, 2008) and another study from the United States [64], are the only ones that have presented separate associations for never-smokers. Wu and colleagues [64], but not Gallagher and colleagues [63], observed a significant dose-response relationship. Only one study has assessed BMD at the lumbar spine. Nawrot and colleagues, observed a non-statistically significant inverse association (p=0.14) between urinary cadmium and BMD at the lumbar spine in in men occupationally exposed [65]. Although several studies have found adverse associations between cadmium exposure and bone, there are a few studies reporting non-significant associations or even null findings [66-69]. Thus, there is a need to clarify the role of cadmium in development of osteoporosis, especially focusing on sites particular susceptible to osteoporotic fractures with high public health importance. Even more important is of course the evaluation of fracture risk. It is also essential to rule out any possible non-cadmium mediated negative effect of tobacco smoking on bone. Although food is the main source of exposure in most people the relation between the dietary cadmium intake and BMD or osteoporosis has never been explored.

2.2.6 Vitamin D, cadmium and bone

The mechanism of cadmium-induced bone damage is not yet fully understood. Two main hypotheses have been suggested: a direct effect on the skeleton possibly through activation of osteoclasts and ii) an indirect effect, via an initial kidney damage, inhibiting the conversion of 25-hydroxy vitamin D (25(OH)D) to 1,25-dihydroxy vitamin D ($1,25(OH)_2D$) [70]. The accumulation of cadmium in the renal cortex has been proposed to interfere with enzymes either directly or indirectly involved in this conversion of 25(OH)D to $1,25(OH)_2D$. In addition, cadmium may decrease the tubular reabsorption of elements necessary for proper bone metabolism, such as calcium, resulting in increased excretion of these elements in urine. Concerning the effect of cadmium on $1,25(OH)_2D$ activation, several experimental studies have shown inconsistent results at cadmium concentrations relevant to human exposure [70]. Even though the limited number of available human studies suggests lower serum $1,25(OH)_2D$ concentrations at high cadmium exposure [71-74], the results are inconclusive. Furthermore, as most of the included subjects were already severely affected by cadmium-induced renal dysfunction [71-74], nothing is known about its involvement in the early onset of bone effects.

2.2.7 Vitamin A, cadmium and bone

Experimental studies have shown that high retinoic acid concentrations and also high concentrations of cadmium seem to give raise to teratogenic effect on limb formation [75].

One study has also shown that cadmium may induce the biosynthesis of retinoic acid *via* up-regulation of genes that are involved in the retinoic acid metabolism as well as an inhibition of enzymes that are involved in the degradation of retinoic acid [76]. Studies on vitamin A in humans have shown inconsistent results with regard to associations with BMD and fracture risk. There is no data in humans on possible combined effect of vitamin A and cadmium on bone.

2.2.8 Risk assessment

For extensive risk assessments on cadmium, see reports from the European Food and Safety Authority (EFSA) [6] and the Joint FAO/WHO Expert Committee on Food Additives (JECFA) [77-80] (*www.fao.org/es/esn/jecfa*).

The JECFA established already in 1972 a provisional tolerable weekly intake (PTWI) for cadmium of 7 µg/kg body weight (bw)/week (60 µg/day for an individual of 60 kg) [77]. In 1988 and 2005, the PTWI was re-evaluated and retained [78, 79]. In 1992, WHO concluded that a urinary cadmium excretion of 10 µg/g creatinine caused tubular proteinuria in 10% in the population [7].

In 2009, a risk assessment was performed by EFSA. A new tolerable weekly intake (TWI) was established; 2.5 µg/kg bw [6] corresponding to 25 µg/d at 70 kg bw. This new TWI was set to keep 95% of the population by the age of 50 below the "reference point" of 1 µg cadmium/g creatinine in urine. The TWI is close to the mean dietary exposure for adults in Europe, but may be exceeded by specific subgroups such as vegetarians, children, smokers and subjects living in contaminated areas. may exceed this TWI with about 2-fold [6].

In 2010, JECFA withdrew their PTWI and expressed a new tolerably monthly intake (PTMI) of 25 µg/kg bw (also corresponding to ≈60 µg/day at 70 kg bw) based on a reference point of 5.4 µg cadmium/g creatinine [80]. Thus, EFSA and JECFA came to very different conclusion on the reference point 1*vs* 5.4 µg/g creatinine (based on the same meta-analysis of urinary cadmium and the tubular effect marker β-2-M). Other differences concerned the statistical methods used to account for uncertainty and variability (JECFA did not account for inter-individual variations), and the methodology for transforming urinary cadmium to dietary cadmium intake. EFSA concluded that their TWI at 2.5 µg/kg bw should be maintained [81, 82].

3 AIMS OF THE THESIS

The overall aim of this thesis was to explore whether long-term low-level exposure to the toxic metal cadmium adversely affects the bone health in women.

The specific objectives were:

- To assess the associations between urinary cadmium and:
  - bone mineral density (BMD) and risk of osteoporosis at sites particularly susceptible to osteoporotic fractures **(Paper I)**
  - risk of fractures, assessed as any first fracture, any first osteoporotic fracture, the most common osteoporotic fracture (distal forearm), and multiple fractures **(Paper I)**

- To determine the association between questionnaire-based dietary cadmium exposure and BMD and risk of osteoporosis and fractures **(Paper II)**

- To explore whether the inverse association between cadmium exposure and BMD is mediated *via* reduced activation of 25(OH)D to $1,25(OH)_2D$, as assessed by the circulating concentrations in serum **(Paper III)**

- To elucidate possible interactions between cadmium and vitamin A on bone, through measurements of biomarkers of exposure, BMD and biochemical markers of bone turnover **(Paper VI)**

4 SUBJECTS AND METHODS

The following is a summary of the study populations and methods used in this thesis. Further details can be found in each respective paper (**I-IV**).

4.1 STUDY POPULATIONS AND SAMPLING

Data from two population-based epidemiological studies are used in this thesis:

- The Swedish Mammography Cohort (SMC) (**Paper I and II**)
- The Women's Health in the Lund Area (WHILA) (**Paper III and IV**)

4.1.1 Swedish Mammography Cohort (SMC)

The Swedish Mammography Cohort (SMC) was established in 1987-1990 with the general aim of assessing the relationships between a number of modifiable factors and the occurrence of several major chronic diseases (http://www.imm.ki.se/smc/). All 90,303 women, born between 1914 and 1948 and residing in two counties (Uppsala and Västmanland) in central Sweden received an invitation to be screened by mammography. Enclosed with this invitation was a six-page questionnaire covering information on dietary habits (food frequency questionnaire, FFQ), weight, height, parity and education; 66,651 (74%) of the women completed the questionnaire [83].

In 1997, a more comprehensive questionnaire was sent out to all 56,030 women still living in the study area, with extended questions on dietary habits (FFQ) and with collection of information on reproductive factors including use of postmenopausal hormones, physical activity, education, smoking history and medical history of certain diseases. A completed questionnaire was obtained from 38,984 women (70% response rate) [83].

4.1.1.1 SMC sub-cohort

In the present thesis (**Paper I and II)**, the association between cadmium exposure and BMD, osteoporosis and fractures were studied in a sub-cohort of the SMC consisting of women living in the town of Uppsala. Since 2003, the women were invited to complete a detailed questionnaire on diet and lifestyle factors, and to undergo a health examination at Samariterhemmet in Uppsala. The examination was conducted after overnight fasting and included total-body BMD measurements (DXA), weight and height measurements, sampling of fat tissue, blood, and from 2004 also morning spot urine.

The recruitment continued until early September 2009, when in total 8,311 women had been invited (65% responded and completed the FFQ). In total, 5,022 women (aged 54-85 years) underwent BMD measurements (60%). For **Paper I and II**, we included women who had been recruited until the end of December 2008 and by that time 4,718 women had undergone BMD measurements, 4,276 had provided urine samples for cadmium analysis and 4,575 women had filled in the questionnaires. Out of these women, 2,820 were below 70 years of age (at time of clinical examination), which was the age limit chosen for the cadmium study in order to avoid an inverse effect of old age on kidney cadmium accumulation [84]. For additional exclusion criteria, see **Figure 3**.

4.1.2 Women's Health in the Lund Area (WHILA)

All women 50 to 59 years of age and living in the Lund area by December 1st, 1995 (*n* = 10,766) were invited to a health screening program (participation rate 64%; n=6,917) [85]. Together with the invitation, the women received a basic questionnaire including questions on previous and present diseases, drug treatment, smoking- and alcohol habits, education, physical activity, working status, parity, months of lactation, and menopausal status [86]. The women underwent measurements of forearm BMD (DXA), weight, height and minimal waist- and maximum hip circumference [85].

4.1.2.1 The cadmium-study in WHILA

In June 1999, when 1,160 women remained to be invited, the study was extended to include health aspects of cadmium exposure; 820 women (aged 54 to 63 years) participated (71%). Out of these women, 813 provided a morning urine sample and 742 women a blood sample [53, 61]. Because urinary cadmium was inversely associated with BMD in these women (Åkesson et al., 2006), we wanted to explore whether this association was mediated *via* a decreased activation in the kidneys of vitamin D, as assessed by the concentration of $1,25(OH)_2D$ in serum (**Paper III**). Further, in order to elucidate a possible interaction between cadmium and vitamin A on the effects on bone (**Paper IV**), we analysed vitamin A (retinol) in serum. Thus, for the purpose of **Paper III and IV** we used, as previously measured and described elsewhere, cadmium in urine and blood and several biochemical markers of bone turnover such as parathyroid hormone (PTH), bone alkaline phosphatase (bALP), and osteocalcin in serum and deoxypyridinoline (DPD) in urine, as well as kidney effect markers: serum cystatin C, urinary *N*-acetyl-β-D-glucosaminidase (NAG) and urinary protein HC (α1-microglobulin) [53, 61]. For exclusion criteria (**Paper III** and **IV)**, see **Figure 3**.

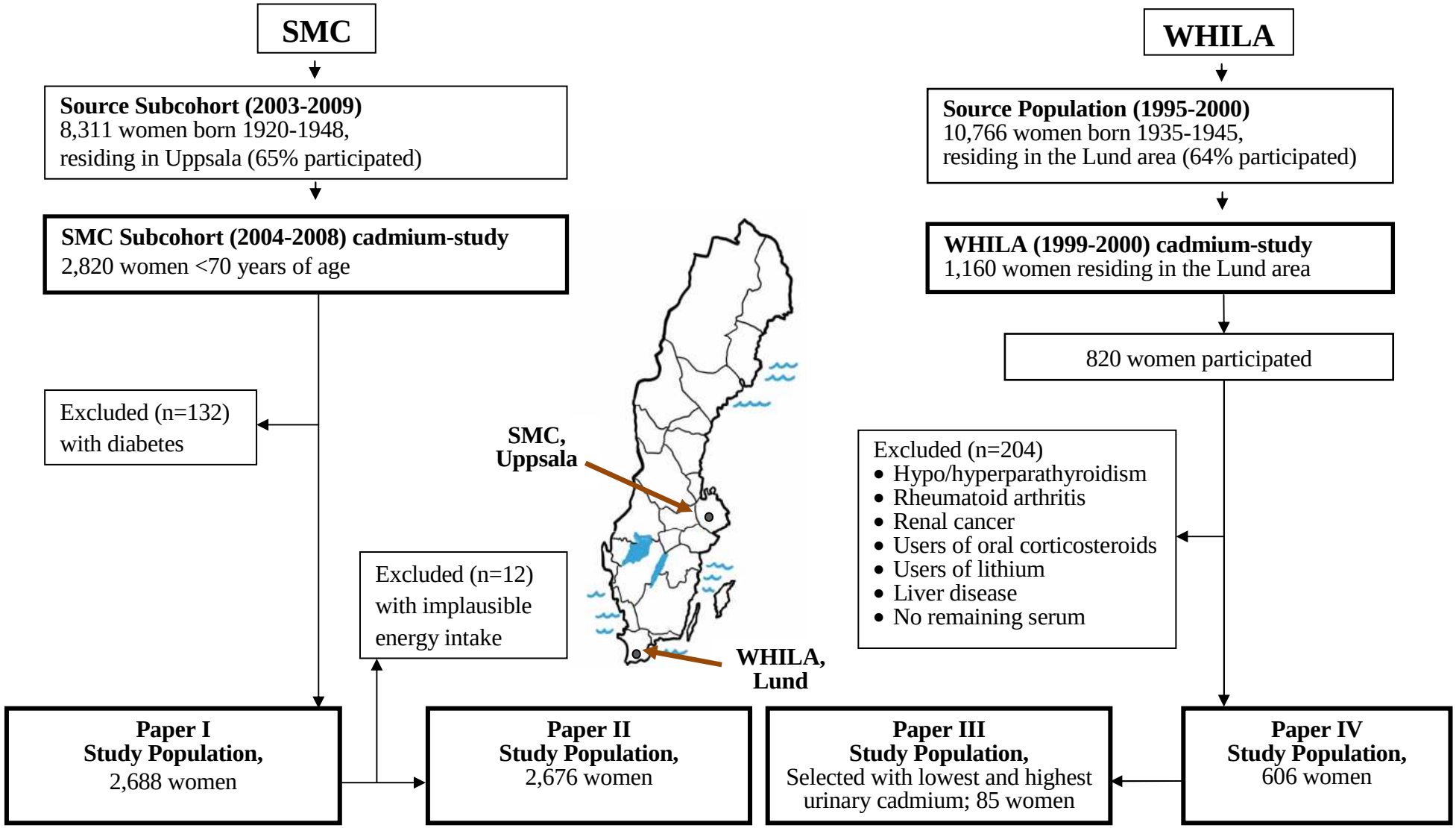

Figure 3. The Swedish Mammography Cohort (SMC) and Women's Health in the Lund Area (WHILA), source populations and study populations for **Paper I-IV**. Figure: ©Magga-Rita.

4.2 BIOMARKERS OF EXPOSURE AND EFFECT

4.2.1 Element analyses (Paper I-IV)

In order to assess the long-term exposure to cadmium we used urinary cadmium, as this reflects accumulation of cadmium in kidneys over decades [42]. In non-smokers urinary cadmium is likely to mainly reflect the dietary cadmium exposure. We also measured calcium and magnesium in urine since they are important for bone structure [10]. In **Paper III**, we also used available data on blood cadmium concentration, mainly reflecting the short-term exposure [53, 61].

The participating women in the SMC-subcohort received by mail a cup (previously tested free from metal contamination) to collect the first voided morning urine sample and a 13-ml polyethylene tube (acid-washed, Sarstedt, Nümbrecht, Germany) for storage of the urine. A detailed sampling instruction was included to minimize the risk of contamination of the urine (**Paper I and II**). The women brought the urine sample to the clinical examination at Samariterhemmet, Uppsala City (SMC). A similar procedure was used for the collection of urine samples in **Paper III and IV** (WHILA).

4.2.1.1 Cadmium analyses

All laboratory utencils for the urinary analysis, such as tubes and tips (polyethylene; Sarstedt, Nümbrecht, Germany) were acid-washed. Urinary cadmium was measured at the Institute of Environmental Medicine (IMM), Karolinska Institutet (KI), using inductively coupled plasma mass spectrometry with a collision/reaction cell operated in helium-mode (ICPMS; Agilent 7500ce, Agilent Technologies, Waldbronn, Germany) [87], measuring cadmium isotope 111 (*m/z* 111) in all samples (**Paper I and II**). Helium mode was used in order to minimize polyatomic interferences; mainly by molybdenum oxide.

For analysis, the urine sample was diluted 1:10 with 1% HNO_3 (nitric acid) (suprapur, Merck, Darmstedt, Germany). In order to evaluate the analytical accuracy for cadmium, several appropriate reference materials were included in the analyses (**Table 1** and **Figure 4**). In general, the results of all the reference materials were in good agreement with the reference values (CV <15%). One exception was, however, the cadmium concentrations of Seronorm OK4636 (Seronorm™ Trace Elements Urine Blank, REF 201305; SERO AS, Billingstad, Norway), which was consistently somewhat lower than the recommended value, also observed by others. However, two *certified* standard reference materials (CRM; National Institute of

Standards and Technology (NIST), Gaithersburg, MD, USA) and an additional Seronorm (101021; aiming at same concentrations as OK4636) were included. All these showed very good agreement with the reference values. The low CRM (reference value 0.059 ± 0.0034 µg/L) aimed at assuring the analytical quality at very low cadmium concentrations. In total only 35 urine samples were below the reference value for this low CRM (<0.059 µg/L). The mean limit of detection (LOD), calculated as 3 x standard deviation (SD) of the mean blank values was 0.002 µg/L, which is the lowest LOD published. No samples had cadmium concentrations below the LOD; the lowest urinary cadmium concentration in the women was 0.02 µg/L. In addition to all the reference material, we also included one "in house" control urine sample (treated in the same way as the urine samples from the women), which showed good repeatability (**Figure 4**). In total, we performed 18 runs (**Table 1**). Altogether, the rigorous quality program and the high analytical precision ensured that the measured urinary cadmium concentrations are valid and that the uncertainty is low even in the low dose range (**Table 1** and **Figure 4**).

In order to account for variation in urine dilution, all cadmium concentrations were adjusted for urinary creatinine (µg/g creatinine; Clinical Chemistry, Västerås Hospital, Västerås, Sweden) as well for urinary density (g/mL) (IMM, KI). Urinary density was measured with refractometer (URICON-NE refractometer, Atago Co., Ltd, Tokyo, Japan). The mean urinary density in the SMC sub-cohort was 1.015 g/mL.

In **Paper III** and **IV** (WHILA), ICPMS (Thermo X7, Thermo Elemental, Winsford, United Kingdom) was also used for cadmium measurements in urine and blood, under strict quality control [53, 61]. These analyses were performed at the Department of Occupational and Environmental Medicine, Lund University, Sweden. The LOD for urinary cadmium was 0.31 µg/L and 0.12 µg/L for blood cadmium [53] with 172 urine (22%) and 3 blood samples (0.4%) below LOD, respectively. For samples below LOD the actual obtained concentrations were used in the calculations, although the concentrations below LOD are measured with a higher uncertainty as compared to those above LOD. In contrast to the ICPMS method in **Paper I and II**, no collision/reaction cell was used. Thus, we cannot rule out possible influence of molybdenum oxide. Urinary cadmium concentrations were adjusted for urinary creatinine (µg/g creatinine) as well as urinary density (mean urinary density of 1.015 g/mL in WHILA).

4.2.1.2 Calcium and magnesium analyses

In **Paper I** (SMC), calcium (*m/z* 43) and magnesium (*m/z* 25) were measured in urine with ICPMS at the same occasion as cadmium. As for cadmium, the same commercial reference materials were used to assure quality-control; see **Table 1** for details. The mean LOD for calcium was 8.0 µg/L and for magnesium 1.1 µg/L. In general, the obtained concentrations were in agreement with the reference values, with the exception of Seronorm 101021 for magnesium, for which the obtained concentrations were approximately 1/3 of the reference value. However, these obtained concentrations were very stable and had a low CV. The reason for the discrepancy is not known but it should be noted that Seronorm references are not certified. As the results for magnesium in all other reference materials were well within the target values, we are confident that we have adequate evidence of accurate analytical results.

In **Paper III** (WHILA), calcium in urine was measured with ICPMS in Lund [53, 61]. The LOD was 1.6 mg/L with 1 sample <LOD.

Table 1. Obtained concentrations (mean±SD) and reference values in urine for the reference material used to ensure quality control (**Paper I).** No reference value is available for the "in house" urine sample.

Reference materials	**n**	**Obtained value**	**Reference value**	**CV (%)**
Cadmium				
NIST, low, CRM (µg/L)	18	0.070 ± 0.0092	0.059 ± 0.0034	13
NIST, high, CRM (µg/L)	18	5.2 ± 0.061	4.7 ± 0.084	1.2
In house control sample (µg/L)	157	0.79 ± 0.05	-	5.8
Seronorm, OK4636 (µg/L)	214	0.23 ± 0.04	0.31 ± 0.05	15
Seronorm, NO2525 (µg/L)	130	4.7 ± 0.19	5.1 ± 0.22	8.0
Seronorm, 101021 (µg/L)	21	0.37 ± 0.03	0.35 ± 0.08	4.1
Calcium				
NIST, low, CRM (µg/L)	18	30 ±3.2	30 ± 2	11
NIST, high, CRM (µg/L)	18	30 ± 2.7	29 ± 2	9.0
In house control sample (µg/L)	157	17 ± 1.5	-	8.8
Seronorm, OK4636 (µg/L)	214	125 ± 10	116 ± 6	8.0
Seronorm, NO2525 (µg/L)	130	118 ± 10	108 ± 4	8.7
Seronorm, 101021 (µg/L)	21	137 ± 12	130 ± 2	8.7
Magnesium				
NIST, low, CRM (µg/L)	18	19 ± 1.9	21 ± 0.2	10
NIST, high, CRM (µg/L)	18	20 ± 1.9	21.2 ± 0.2	9.5
In house control sample (µg/L)	157	15 ± 1.2	-	8.0
Seronorm, OK4636 (µg/L)	214	86 ± 8.9	89 ± 4	10
Seronorm, NO2525 (µg/L)	130	56 ± 3	54 ± 3	9.0
Seronorm, 101021 (µg/L)	21	59 ± 5.3	185 ± 40	9.1

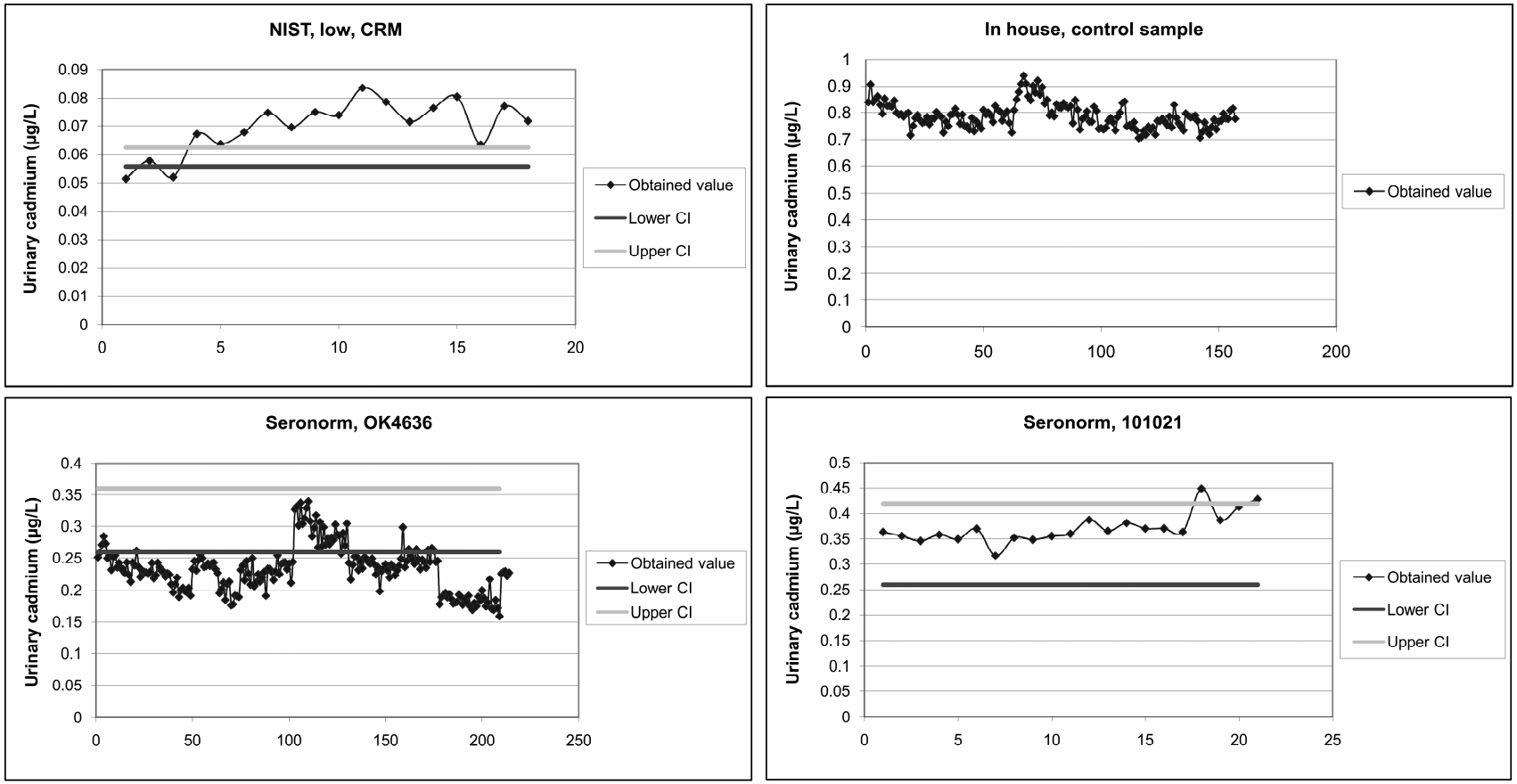

Figure 4. Obtained cadmium results for low CRM, “in house” control sample, Seronorm OK4636 and Seronorm 101021.

4.2.2 Vitamin D analyses (Paper III)

In **Paper III** (WHILA), we assessed 1,25$(OH)_2$D in serum as a marker of the activation of 25(OH)D to 1,25$(OH)_2$D using an enzyme immunoassay (EIA). Immunoextraction was followed by quantification (IDS OCTEIA Ltd., Boldon, United Kingdom). These analyses were performed at IMM, KI. The EIA quantified the two forms 1,25$(OH)_2D_3$ (cross-reactivity 100%) and 1,25$(OH)_2D_2$ (cross-reactivity 39%). The sensitivity was 6 pmol/L and the assay ranged from 4 to 500 pmol/L. Because of the cumbersome and expensive assessment of 1,25$(OH)_2$D, we selectively analysed 45 women with low urinary cadmium concentrations and 40 women with high urinary cadmium concentrations. In addition, we analysed 25(OH)D (EIA) in 42 women (n=22 with low urinary cadmium and n=20 with high urinary cadmium) in order to exclude a possible effect on the concentration of 1,25$(OH)_2$D due to differences in vitamin D status [26]. Samples for vitamin 25(OH)D analyses were collected during winter season to avoid major influence on the concentration of variations in sunlight exposure [88]. The corresponding cross-reactivity for 25(OH)D was 100% for 25$(OH)D_3$ and 75% for 25$(OH)D_2$. The results of the provided control samples from the manufacturer were within the recommended range**.** One "in house" serum control sample was included in a similar manner as the analysis of urine samples. The CV for the three control samples, including the" in house" sample, for 1,25$(OH)_2$D were 6.2%, 7.1% and 9.2% and for 25(OH)D 8.1%, 4.7% and 5.9%, indicating good analytical accuracy.

4.2.3 Retinol analysis (Paper IV)

In **Paper IV** (WHILA), serum retinol was measured in order to evaluate the retinol status. These analyses were performed at IMM, KI. Since vitamin A is sensitive to degradation from UV-light, all the windows and strip-lights were shielded with special UV-filters. The concentrations of retinol in serum were assessed in 606 women (WHILA) by high pressure liquid chromatography (HPLC) followed by fluorescence detection (excitation 325 nm; emission 475 nm) [89]. Retinyl acetate and retinol (external standards) were used for quantification. The stock solution, including retinyl acetate and retinol was checked every morning for degradation and samples from the stock solution was prepared fresh every morning. All the samples were analysed in duplicates. The CV for the "in house" control sample was 13%, indicating a satisfactory analytical accuracy.

4.2.4 Bone and kidney markers

We used the previously measured bone turnover markers parathyroid hormone (PTH), bone alkaline phosphatase (bALP), osteocalcin and urinary deoxypyridinoline (DPD) and the kidney effect markers serum cystatin C, *N*-acetyl-β-D-glucosaminidase (NAG) and urinary protein HC (α1-microglobulin) [53, 61].

4.3 DIETARY ASSESSMENT

A comprehensive food-cadmium database (recipe-based) has been constructed at IMM, KI based on the cadmium content present in almost all foods available on the Swedish market [38, 90]. With few exceptions, the food cadmium data were obtained from the National Food Administration (Uppsala, Sweden). The daily cadmium intake was estimated by multiplying the cadmium content with the consumption frequency and specific portion sizes, based on the FFQ and the food-cadmium database. The FFQ has previously been validated against weighted food records (Pearson correlation coefficients of 0.5-0.8 for the major cadmium containing food groups; Wolk, unpublished data).

For **paper II**, the cadmium intake estimated from the FFQ in 1997 was used to assess the association between dietary cadmium exposure and bone effects. The FFQ from 2004-2008 completed at the time of the urine sampling was not considered since it does not to reflect the relevant time period of the exposure in relation to the bone measurements. In addition, this enabled the risk of fractures to be assessed prospectively with follow-up starting in 1997 (**Paper I and II**; SMC).

The intakes of calcium, magnesium, fiber and iron, which all may influence bone health, were also obtained from the FFQ. Cadmium, calcium, magnesium, fiber and iron were energy adjusted (to 1700 kcal; mean in the cohort) by using the residual method [91]. Energy-adjustment is consistently used in nutritional epidemiology and is a way to compensate for possible under- or over reporting of intake. The approach is based on the concept that the composition of the diet, independent of the energy intake, is of primary interest in relation to disease risk. Energy-adjustments can limit misclassification of the cadmium/nutrient intake due to differences in body size, physical activity etc. [91].

4.4 BONE MEASUREMENTS

4.4.1 BMD

In **Paper I and II** (SMC), we used data on BMD (g/cm^2) measured with DXA (DPX Prodigy, Lunar corp., Madison, WI, USA) at Samariterhemmet, Uppsala [92]. BMD was measured at the total body, femoral neck, total hip and lumbar spine (vertebrae L_2-L_4) (**Figure 5**). In **Paper I**, 2.0% of the women and 1.6% of the women in **Paper II** had only one-sided measurements done at the femoral neck due to metal implant (e.g. prosthesis or plate). However, in order to keep as many women as possible in the analyses, these women were also included. In all other women, the mean BMD of both sides were used.

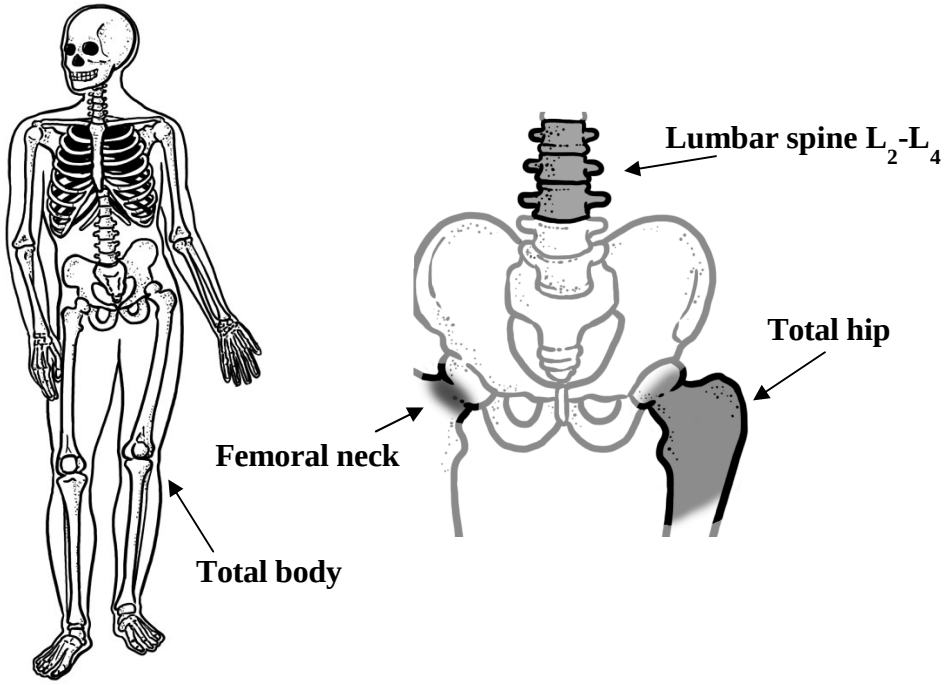

Figure 5. Sites measured at the BMD measurements at Samariterhemmet, Uppsala (**Paper I and II**; SMC). Figure: ©Magga-Rita.

4.4.2 Osteoporosis

The obtained BMD was compared to the mean value in a reference population (aged 20 to 29 years) from the National Health and Nutrition Examination Survey (NHANES) [93]. Osteoporosis was defined as a *T*-score <-2.5. The one and the same technician performed the BMD measurements, and a phantom was used every day for internal validation. The precision error for the BMD measurements was very low (0.8-1.5%). The long-term CV precision for lumbar spine was <1%. At the DXA measurements,

total fat mass and total lean body mass were also measured with high precision (for details, see **Paper I**).

In **Paper III and IV** (WHILA), BMD was measured at the non-dominant distal forearm (at the 8 mm position), using DXA (DXT 200; Osteometer MediTech, Inc., Hawthorne, CA, USA) as previously reported [61, 85, 86]. As for SMC, the one and the same technician in the WHILA study performed all the measurements and a phantom was used for daily calibration.

4.4.3 Fractures

We obtained information of eligible fractures for **Paper I and II** (SMC) by computerized linkage of the cohort, using the personal identification number given to all Swedish citizens, with the regional hospital diagnosis registries and to the National Patient Registry, thereby covering both outpatient and inpatient treated fractures [94]. The codes used were from ICD-10 and included S12, S22, S32, S42, S52, S62, S72, S82 and S92 (all fractures) [94]. The classical osteoporotic fractures were considered as fractures at the hip, spine, distal forearm, proximal humerus, and pelvis; the most common first osteoporotic fracture was distal forearm. A validated method for multiple fractures (that is, multiple incident fracture occasions) was used to identify the incident injuries from the resubmissions [95].

4.5 ETHICS

Oral or written informed consent regarding participation was obtained from all women. The ethical committees at Lund University in Lund, Sweden and the Regional Ethical Review Board in Stockholm, Sweden approved the studies. The results of the BMD measurements were reported to the women at their clinical visit and those women with low BMD were directed to practitioners for further investigation.

4.6 STATISTICAL METHODS AND ANALYSES

For univariate associations, Spearman rank correlation coefficient (r_s) was used for continuous variables and Kendall's tau_b for categorical variables. Mann-Whitney U-test, Kruskal-Wallis or χ^2-test were used to assess the differences between independent groups. In order to account for the influence of other factors we performed multivariable-adjusted regression analyses. The residual analysis indicated no major deviation from a linear pattern in the linear regression. Odds ratio (OR) and its 95%

confidence interval was achieved by using logistic regression. Multicollinearity was tested with tolerance (>0.1) and variance inflation factor (VIF, <10) for both linear and logistic regression. Binary logistic regression models were tested for Hosmer-Lemeshow (>0.05) and ordinal logistic regression for a Pearson goodness-of-fit and proportional odds assumption (>0.05).

In **Paper I** (SMC), several different statistical analyses were used to assess the relationship between urinary cadmium and BMD, osteoporosis and fractures. We used linear regression analyses, analysis of covariance (ANCOVA), and binary- and ordinal logistic regression analyses. Additionally, restricted cubic spline analyses (with three "knots") were used in order to make the model flexible, and also to be able to graphically show the association between urinary cadmium and risk of osteoporosis [96]. Urinary cadmium was treated either as a continuous variable (per 1 µg/g creatinine or per 0.42 µg/g creatinine (=2 SD) increment) or as a categorized variable into predefined groups or into tertiles. The predefined categories were <0.50, 0.50-0.75 and ≥0.75 µg/g creatinine to facilitate comparison with previous studies [57, 63] using concentrations of <0.50 µg/g creatinine as the reference category. Because few women had urinary cadmium >1 µg/g creatinine (n=46) in SMC, a middle exposure category (0.50-0.75), which in contrast to previous studies had to be included. Trend across categories was tested using the median urinary cadmium within categories as a continuous variable in order to avoid giving too much weight to potential outliers.

In **Paper II** (SMC), estimated dietary cadmium exposure was either continuously treated as per 10 µg/day increment or in 2 categories; below (low) or above the median (high) dietary cadmium intake (< and ≥13 µg/day). The relationship between dietary cadmium and BMD, osteoporosis and fractures were analysed with linear and binary logistic regression analyses**.** Dietary factors important for bone health and cadmium bioavailability (calcium, magnesium, iron and fiber) were additionally included in order to examine if these would attenuate the associations between dietary cadmium and BMD and fractures. The combined effect of dietary cadmium (low or high) and urinary cadmium (<0.50 or ≥0.50 µg/g creatinine) were assessed by combining the high dietary cadmium category with the high urinary cadmium category. Women with low dietary cadmium intake and low urinary cadmium excretion constituted the reference category while the remaining women were categorized into the intermediate category.

In **Paper III** (WHILA), univariate analyses evaluated the relationship between $1,25(OH)_2D$ and several exposure- and effect markers. Linear regression analyses were used to evaluate the relationship between $1,25(OH)_2D$ and urinary- or blood cadmium.

In **Paper IV** (WHILA)**,** univariate and linear regression analyses were used. A potential non-linear association between serum retinol and BMD was evaluated by additionally adding squared serum retinol to the model. In addition, a multivariable-adjusted scatterplot was calculated in order to visually observe a possible non-linear trend between serum retinol and BMD. Urinary cadmium was subsequently added to the statistical models evaluating the association between retinol and BMD and bone turnover markers. To evaluate a possible combined effect, serum retinol and urinary cadmium were categorized into 2 groups: below (low) and above (high) the median (< and ≥1.9 µmol for serum retinol; < and ≥ 0.66 nmol/mmol creatinine for urinary cadmium). Women having the lowest serum retinol and the highest urinary cadmium had the lowest BMD and therefore constituted the reference category, while the remaining women were categorized into high/high, low/low and high/low concentrations of retinol and urinary cadmium, respectively. This association was analyzed with linear regression.

The covariates included in the statistical models in **Paper I-IV** were selected if associated with both the exposure and the outcome, changing the estimate for the exposure in the multivariable-adjusted model by more than 10% and/or are traditionally known protective- or risk factors for BMD and fractures. For more details on the included variables; see the separate papers.

The statistical analyses were carried out using SPSS (PASW), version 14 or 18.0 (SPSS Inc., Chicago, IL, USA), Stata 10.1 (Stata Corporation, Inc., Collage Station, TX, USA) and Microsoft Excel 2010 (Microsoft Corporation, Redmond, WA, USA). All tests were two-sided, and a $p<0.05$ was considered statistically significant.

5 RESULTS AND DISCUSSION

This section is a summary and discussion of the main results and conclusions that can be drawn. For further details, the reader is referred to the separate Papers (**I-IV**). Some additional results are included that are not reported in the Papers.

5.1 CADMIUM EXPOSURE

5.1.1 Biomarkers of exposure

The median urinary cadmium concentration in the 2,688 women from the SMC (**Paper I-II**) was 0.34 µg/g creatinine (second black line from the bottom in **Figure 6**). The median concentration in 606 women in the WHILA study (**Paper IV**) was 0.66 µg/g creatinine (second grey line from the bottom; p<0.001 for difference between studies). The exposure categories used in **Paper I, II and IV** are also indicated in **Figure 6.** In the WHILA study, 70% of the women had urinary cadmium concentrations >0.50 µg/g creatinine as compared to 23% in the SMC. The corresponding percent for cadmium concentrations above >1.0 µg/g creatinine was 20% and 0.2%, respectively.

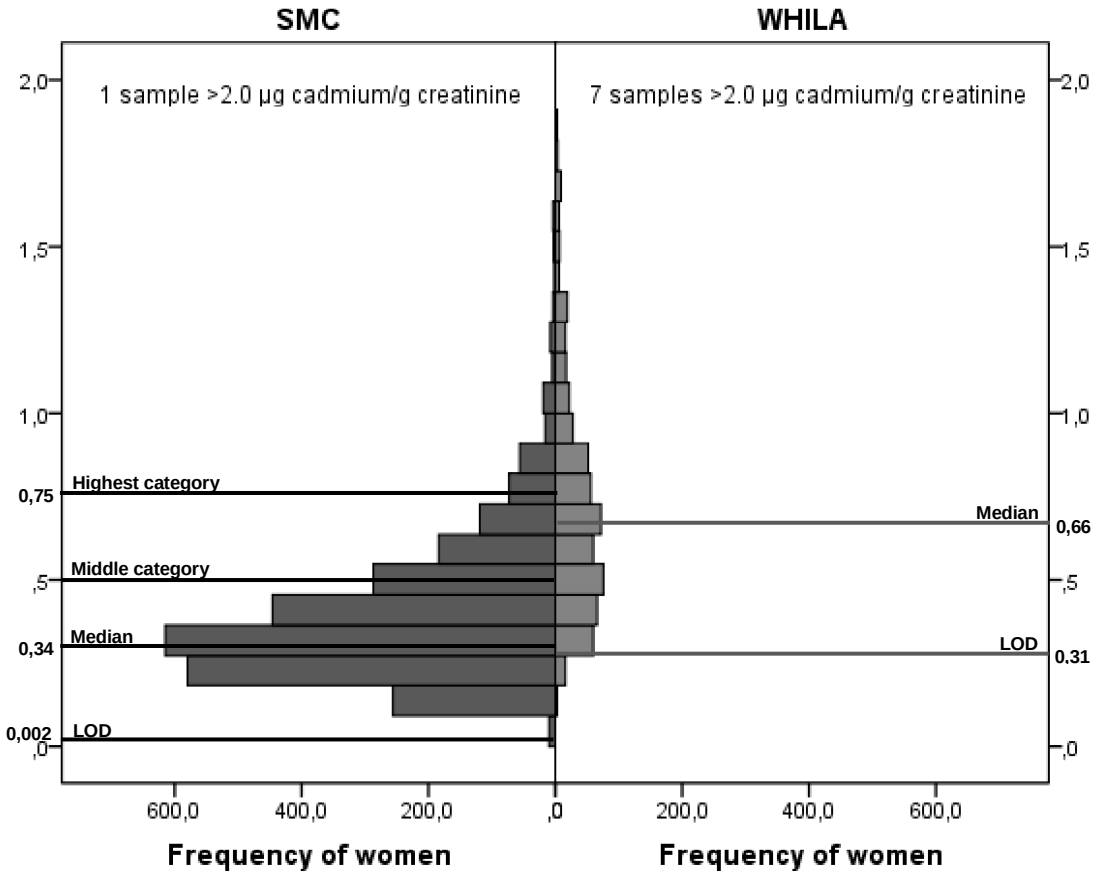

Figure 6. Comparison of urinary cadmium (µg/g creatinine) in the SMC and WHILA studies (all women).

The differences in urinary cadmium concentrations between the studies could be attributed to a real difference in exposure with a higher long-term body accumulation of cadmium in women in Lund (Southern Sweden) than in Uppsala (lower part of Central Sweden). On the other hand, alternative explanations could at least in part contribute to the observed differences. The women in the SMC were somewhat older than the women in the WHILA study, thus it cannot be excluded that an age-related decline in kidney cadmium accumulation [84], eventually resulting in slightly lower urinary cadmium excretion, has occurred. Moreover, the LOD was substantially lower in the SMC (0.002 µg/L; first black line in **Figure 6**) than in the WHILA study (0.31 µg/L; first grey line in **Figure 6**), and polyatomic interferences of molybdenum oxide were only removed in the cadmium analyses in SMC. Thus, analytical differences may also contribute to the observed differences.

The mean urinary cadmium concentration in SMC is similar to that observed in men from an area in Eastern Sweden considered to be environmentally contaminated [57, 97]; while the mean urinary cadmium concentration in the WHILA study is similar to that of the women in the same study [57, 97]. The concentrations are, however, lower in both SMC and WHILA than those observed in Belgium [55], USA (NHANES) [63], and much lower than those in Japan [42]. However, as urinary cadmium also depends on sex, age and smoking habits, these aggregated data-comparisons are not always meaningful.

Among never-smokers, the urinary cadmium concentrations were, as expected; lower than among all women (**Figure 7**), due to the inclusion of smokers among all women. The median cadmium concentration in never-smoking women in the SMC was 0.29 µg/g creatinine and in the WHILA study 0.55 µg/g creatinine ($p<0.001$ for difference). In the WHILA study, 32% of never-smoking women had cadmium concentrations >0.50 µg/g as compared to 6% in the SMC.

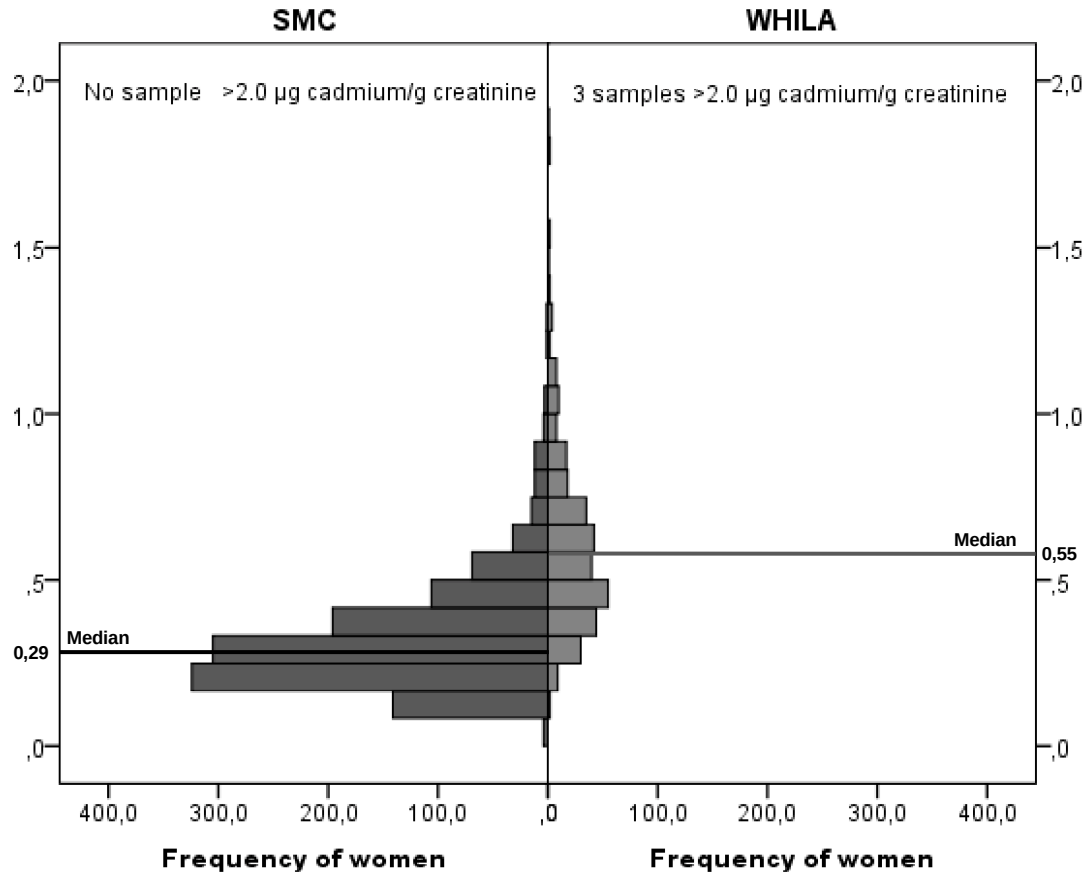

Figure 7. Comparison of urinary cadmium (µg/g creatinine) in the SMC and WHILA study among never-smoking women.

Blood cadmium was only measured in the WHILA study (**Paper III**). The median blood cadmium concentration was 0.38 µg/L among all women and 0.31 µg/L among never-smoking women, which is similar to that observed in Swedish twins (aged 49 to 92 years; mean 68 years) [98] and in women in Southern Sweden (50-59 years) [99], but higher than in Northern Sweden [99, 100]. The blood cadmium concentrations among never-smoking women were also similar to those found in younger women as well as in women in the same age range in Western and Southern Sweden [39, 40] and in Norway [101]. The concentration of cadmium in blood and urine were correlated; r_p=0.55; p<0.001 (n=593).

5.1.2 Dietary cadmium intake

The mean estimated dietary cadmium intake *via* the FFQ was 13 µg/day (range 3-29 µg/day) **(Paper II**), which is in the same range as those observed in Europe and USA (average between 8 and 25 µg/day) [39, 40, 102-108]. The EFSA set a tolerable weekly intake (TWI) of 2.5 µg cadmium/kg body weight to avoid cadmium-induced kidney effects in the general population [6]. In this study (**Paper II**), the estimated average

intake was 1.4 day (range 0.36-3.6) µg cadmium/kg body weight and week, and only 0.7% of the women exceeded the TWI.

5.2 CADMIUM AND BONE

5.2.1 Cadmium and BMD

In **Paper I and II** (SMC), we measured BMD at the total body, femoral neck, total hip and lumbar spine (L_2-L_4). Several covariates were significantly associated with BMD. The most pronounced associations involving all BMD sites were those for age, height, total fat mass, lean body mass, and use of postmenopausal hormones ($p \leq 0.001$). Thus, BMD decrease with age and increase with height, total fat mass, lean body mass and use of postmenopausal hormones ($p \leq 0.002$ for all).

Urinary cadmium (as a continuous variable) was in the crude analyses as well as after multivariable-adjustments significantly inversely associated with BMD at all the sites measured (**Figure 8**). The only exception was the association with BMD at the lumbar spine, which after multivariable-adjustment fell out of statistical significance ($p=0.088$). Although no women had spondylosis at the lumbar spine, which may result in falsely elevated BMD attenuating the observed associations, the general variability of BMD at the lumbar spine was to some extent higher (mean ± SD; 1.13±0.18 g/cm^2) than that at the femoral neck (0.89±0.12 g/cm^2) and total hip (0.94±0.12 g/cm^2) in accordance with studies in twins [109]. The higher variability may explain the somewhat weaker multivariable-adjusted association and lower explained variance (14%) in the analysis of BMD at the lumbar spine, as compared to the hip.

Our study is the first to ever assess the associations between urinary cadmium and BMD at the total body, and the first to assess the association with BMD at the lumbar spine in the general population. Only two previous studies (in occupationally exposed men) have assessed the association between urinary cadmium and BMD at the lumbar spine [110, 111]. Järup and colleagues however reported null associations (n=43) [110], while Nawrot and colleagues observed a non-significant inverse association ($p=0.14$; n=83) [111].

Our results (**Paper I**) are in line with the inverse associations observed between urinary cadmium and BMD in previous studies [55, 57, 60, 61, 63]. However, it should be

noted that all these studies, with the exception of the NHANES-study [63], restricted the BMD measurement to the forearm.

In **Paper I**, the strongest correlation was observed at the total body, followed by total hip (**Figure 8**). Interestingly, both the age-adjusted as well as the multivariable-adjusted regression coefficients for urinary cadmium were fairly similar between the different sites measured, supporting the consistency of our findings.

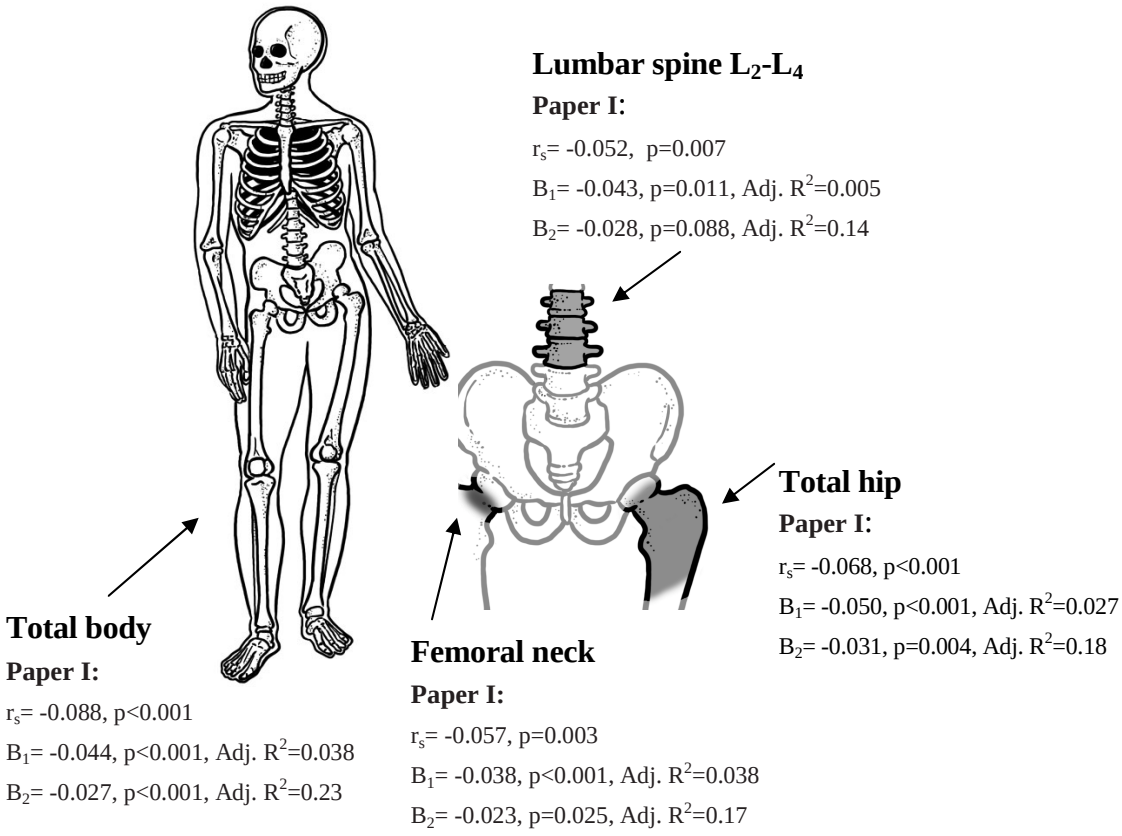

Figure 8. The association between urinary cadmium and BMD at the different sites expressed as crude (Spearman rank correlation coefficient; r_s), age-adjusted regression coefficients (B_1) and multivariable-adjusted regression coefficients (B_2). B_2 was adjusted for age, education, height, total fat mass, lean body mass, parity, ever use of postmenopausal hormones, ever use of corticosteroids, physical activity, alcohol intake, smoking, inflammatory joint diseases, kidney diseases, liver diseases, and malabsorption. Figure: ©Magga-Rita.

The most pronounced inverse association (lowest regression coefficient, B_2= – 0.031) between urinary cadmium and BMD was observed at the total hip, with a higher content of cortical bone as compared to the femoral neck (B_2= – 0.023). This may indicate that cortical bone could be more affected than trabecular bone. The NHANES study, based on women 50 to 90 years of age, is the only previous study performed in

the general population that has measured BMD at the femoral neck and total hip in relation to cadmium exposure [63]. In line with the result in the present study (**Paper I**), the NHANES study observed inverse associations between urinary cadmium and BMD at these two sites with some slight indication of stronger inverse association at the total hip than at the femoral neck [63]. Similarly, Nawrot et al, demonstrated a more pronounced inverse association at the total hip than at the femoral neck in occupationally exposed men (p=0.03 and p=0.11, respectively) [65]. However, also sites including higher content of trabecular bone showed inverse associations with bone (**Figure 8**). Involvement of both bone tissues is supported by animal studies (see e.g. [70, 112-116]. Cadmium enhanced the stiffness in cortical bone and decreased the elasticity in trabecular bone, making the bone more prone to fractures [115]. Female animals seem to be more vulnerable to cadmiums effect on bone than male ones [70]. Aging itself makes the bone stiffer and less elastic [117, 118].

There was no indication of a non-linear association between urinary cadmium and BMD at the total body (**Figure 9**); as also was the case at the femoral neck, total hip and lumbar spine. However, in order to understand if the effect varied at different exposure levels, we also assessed the associations with BMD using categorized urinary cadmium (**Table 2** and **Figure 10**).

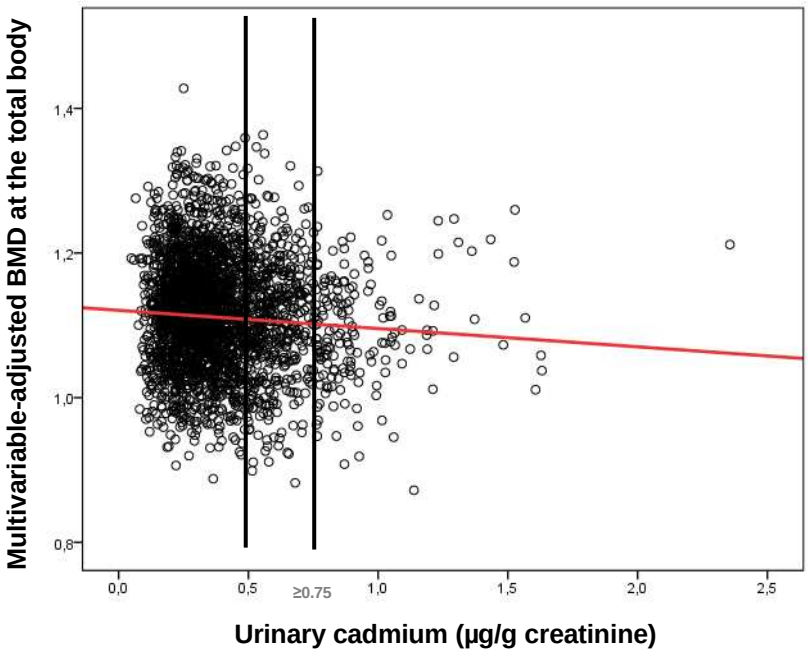

Figure 9. Multivariable-adjusted BMD at the total body. Vertical lines display the cutoffs for the different exposure categories (<0.50, 0.50-0.75 and ≥0.75 µg/g creatinine) used in **Paper I**.

Table 2 and **Figure 10** show the associations between categorized urinary cadmium and BMD at the various sites (results not presented in **Paper I**). The highest exposure category (≥0.75 µg/g creatinine) was significantly inversely associated with BMD at all sites, while the second highest exposure category (0.50-0.75 µg/g creatinine) was significantly associated with BMD at the total body and lumbar spine and borderline significant at the total hip. The results were consistent when using tertiles of urinary cadmium, instead of the chosen cutoffs, supporting the robustness and consistency of our findings.

Table 2. Multiple linear regression coefficients and their 95% confidence intervals (CI) of categorized urinary cadmium in relation to BMD (g/cm^2) at the total body, femoral neck, total hip and lumbar spine.

BMD		Regression coefficient (95% CI)	*p*-value	Adjusted R^2
Total body (TB)				
Urinary cadmium	<0.50	Reference	-	0.23
(µg/g creatinine)[a]	0.50-0.75	-0.013 (-0.021; -0.0044)	0.003	
	≥0.75	-0.027 (-0.039; -0.014)	<0.001	
Femoral neck (FN)				
Urinary cadmium	<0.50	Reference	-	0.17
(µg/g creatinine)[a]	0.50-0.75	-0.009 (-0.020; +0.0023)	0.12	
	≥0.75	-0.032 (-0.049; -0.015)	<0.001	
Total hip (TH)				
Urinary cadmium	<0.50	Reference	-	0.18
(µg/g creatinine)[a]	0.50-0.75	-0.012 (-0.023; +0.0010)	0.052	
	≥0.75	-0.042 (-0.059; -0.024)	<0.001	
Lumbar spine (LS)				
Urinary cadmium	<0.50	Reference	-	0.14
(µg/g creatinine)[a]	0.50-0.75	-0.019 (-0.037; -0.00097)	0.039	
	≥0.75	-0.031 (-0.058; -0.0041)	0.024	

[a] Multivariable-adjusted for age, education, height, total fat mass, lean body mass, parity, use of postmenopausal hormones, ever use of corticosteroids, total physical activity, smoking status, alcohol intake, inflammatory joint diseases, kidney diseases, liver diseases, malabsorption.

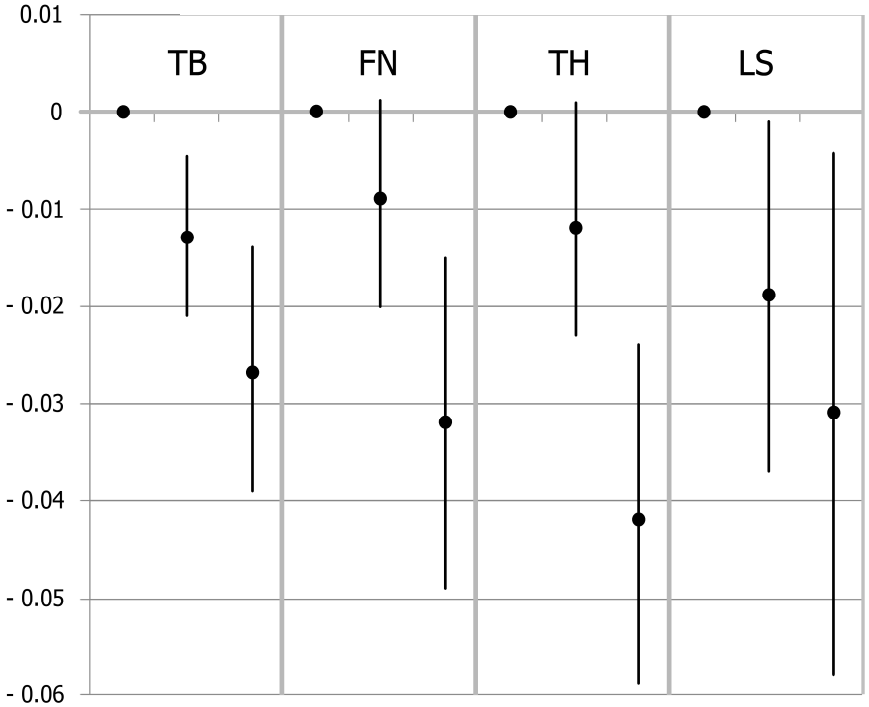

Figure 10. Multiple linear regression coefficients and 95% CI of categorized urinary cadmium in relation to BMD (g/cm^2) at the total body (TB), the femoral neck (FN), the total hip (TH) and the lumbar spine (LS).

Based on categorized multivariable-adjusted urinary cadmium, we estimated the magnitude of difference in BMD between the lowest and the highest exposure groups. As shown in **Figure 11A**, the adjusted mean BMD was lowest in the highest exposure category. The magnitude of the difference in BMD, moving from the lowest urinary cadmium category (<0.50 µg/g creatinine) to the highest (≥0.75 µg/g creatinine), was similar to that observed for a 5-11 years increase in age (for further details, see **Paper I**). This suggests that the cadmium-associated differences observed in BMD are relevant in a clinical perspective. The adjusted mean of BMD for never-smoking women are shown in **Figure 11B**.

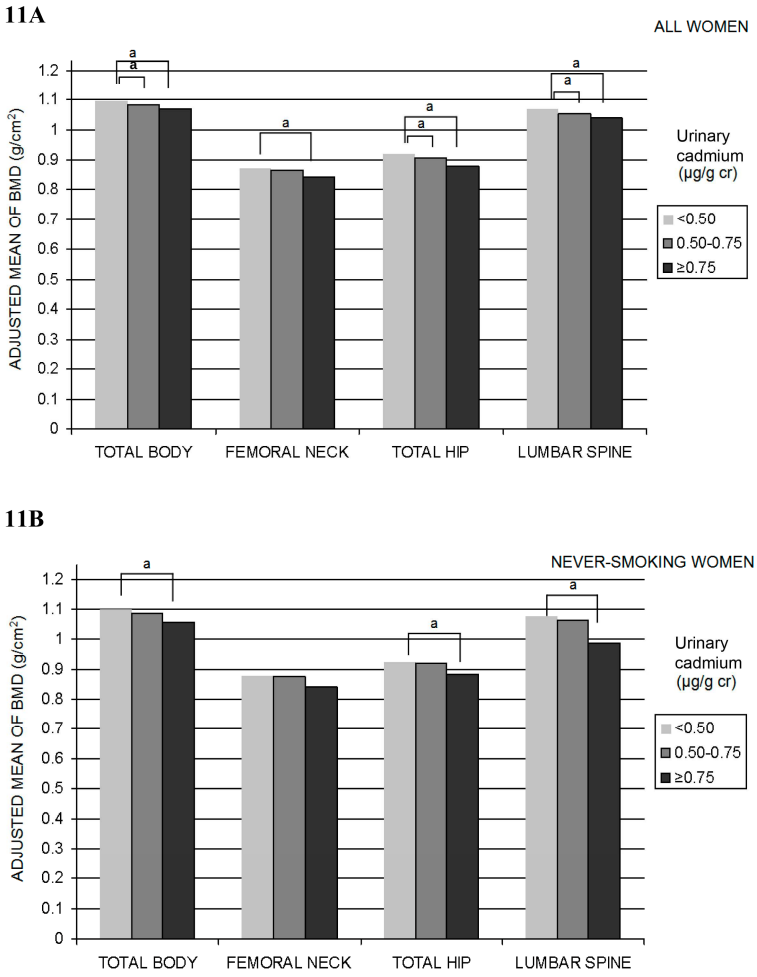

Figure 11. Adjusted mean of BMD based on categorized urinary cadmium among all women and never-smoking women (**Paper I**).

In the general non-smoking population, the diet is the most important source of cadmium exposure [6, 42]. **Paper II** is the first study to assess the associations between estimated dietary cadmium intake and BMD. After multivariable-adjustment, dietary cadmium intake was inversely associated with BMD at the total body and lumbar spine, but not with BMD at the femoral neck. Interestingly, after further adjustments for dietary factors known to be important for bone health (i.e. calcium, magnesium and iron) [119], and for cadmium bioavailability (i.e. iron and fiber), the associations became more pronounced, also at the femoral neck (**Table 3**). This may indicate that there exists a possible risk-benefit association between the dietary exposure of cadmium and the consumption of food considered healthy with a high content of calcium, magnesium, iron and fiber. A risk-benefit relationship has previously been demonstrated for fruit and vegetable consumption in the association between dietary cadmium intake and risk of fractures [107].

We performed sub-group analyses restricted to never-smokers in order to explore whether tobacco smoking confounded our results. However, this restriction did not attenuate our associations, indicating that the associations were attributable to dietary cadmium intake alone (**Table 3,** see below).

As observed in **Figure 12** (**Paper II)**, almost identical multivariable-adjusted linear associations were observed for urinary cadmium as for dietary cadmium exposure in relation to BMD at the total body. This finding provides important support for the utility of questionnaire-based estimated dietary cadmium exposure in large-scale epidemiological studies.

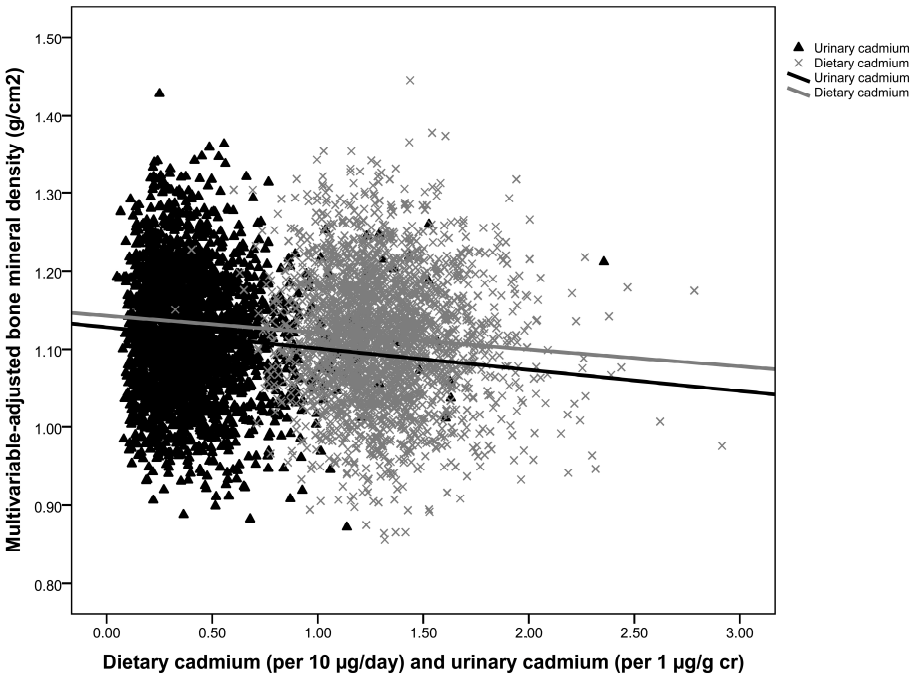

Figure 12.Multivariable-adjusted linear regression for dietary cadmium intake (per 10 µg/day-increment) (grey) and for urinary cadmium (per 1 µg/g creatinine-increment) (black) in relation to total-body BMD.

Table 3. Multiple linear regression based on continuous (per 10 µg/day-increment) dietary cadmium intake and 95% CI in relation to BMD (g/cm^2) at the total body, femoral neck and lumbar spine in all women and in the subgroup of never-smokers.

	Bone mineral density					
	All women, n=2676			**Never-smokers, n=1220**		
	Regression coefficient (95% CI)	***p*-value**	**Adj R^2**	**Regression coefficient (95% CI)**	***p*-value**	**Adj R^2**
Total body						
Dietary cadmium[a]	-0.008 (-0.020 to +0.005)	0.25	0.03	-0.011 (-0.031 to +0.008)	0.26	0.02
Dietary cadmium[b]	-0.012 (-0.024 to -0.00026)	0.045	0.16	-0.020 (-0.038 to -0.001)	0.036	0.14
Dietary cadmium[c]	-0.021 (-0.038 to -0.005)	0.01	0.16	-0.026 (-0.052 to -0.001)	0.04	0.14
Femoral neck						
Dietary cadmium[a]	0.001 (-0.016 to +0.017)	0.94	0.034	-0.004 (-0.029 to +0.022)	0.78	0.02
Dietary cadmium[b]	-0.001 (-0.017 to +0.015)	0.89	0.12	-0.009 (-0.033 to +0.016)	0.49	0.09
Dietary cadmium[c]	-0.018 (-0.040 to +0.004)	0.11	0.12	-0.021 (-0.054 to +0.013)	0.22	0.09
Lumbar spine						
Dietary cadmium[a]	-0.031 (-0.058 to-0.005)	0.02	0.004	-0.041 (-0.081 to -0.001)	0.044	0.003
Dietary cadmium[b]	-0.038 (-0.063 to -0.012)	0.004	0.1	-0.054 (-0.093 to -0.015)	0.003	0.09
Dietary cadmium[c]	-0.058 (-0.093 to -0.023)	0.001	0.1	-0.068 (-0.12 to -0.014)	0.013	0.08

Adj R^2: Adjusted variance for the total model

[a] Age-adjusted estimate for dietary cadmium intake

[b] Multivariable-adjusted for age (years), education (≤9 and >9 years; yes/no), body mass index (kg/m^2), ever use of postmenopausal hormones (yes/no), total physical activity (MET-hours/day), smoking status (never/ever), alcohol intake (g ethanol/day), inflammatory joint diseases (yes/no)

[c] Multivariable-adjusted model, additionally adjusted for dietary intake of calcium (mg/day), magnesium (mg/day), iron (mg/day), fiber (g/day)

In additional analyses we assessed the combined effect of having high dietary and high urinary cadmium. As tobacco smoking may confound these analyses, we also performed separate analyses among never-smokers. In this combined analysis the inverse association between exposure and BMD at all sites was even more pronounced (**Figure 13),** as compared to the separate analyses (**Paper I and II**). As there were no differences in dietary or urinary cadmium in the different exposure categories between the separate and combined analyses (**Table 4**), the result indicate an underestimation of the association between cadmium and bone in the separate analyses. Thus, each of the single markers underestimated the risk indicating some exposure misclassification. The regression coefficients were slightly stronger in the sub-group of never-smokers as compared to those observed in all women (**Figure 13**), indicating that the lower BMD was attributed to cadmium from non-tobacco sources (i.e. mainly food).

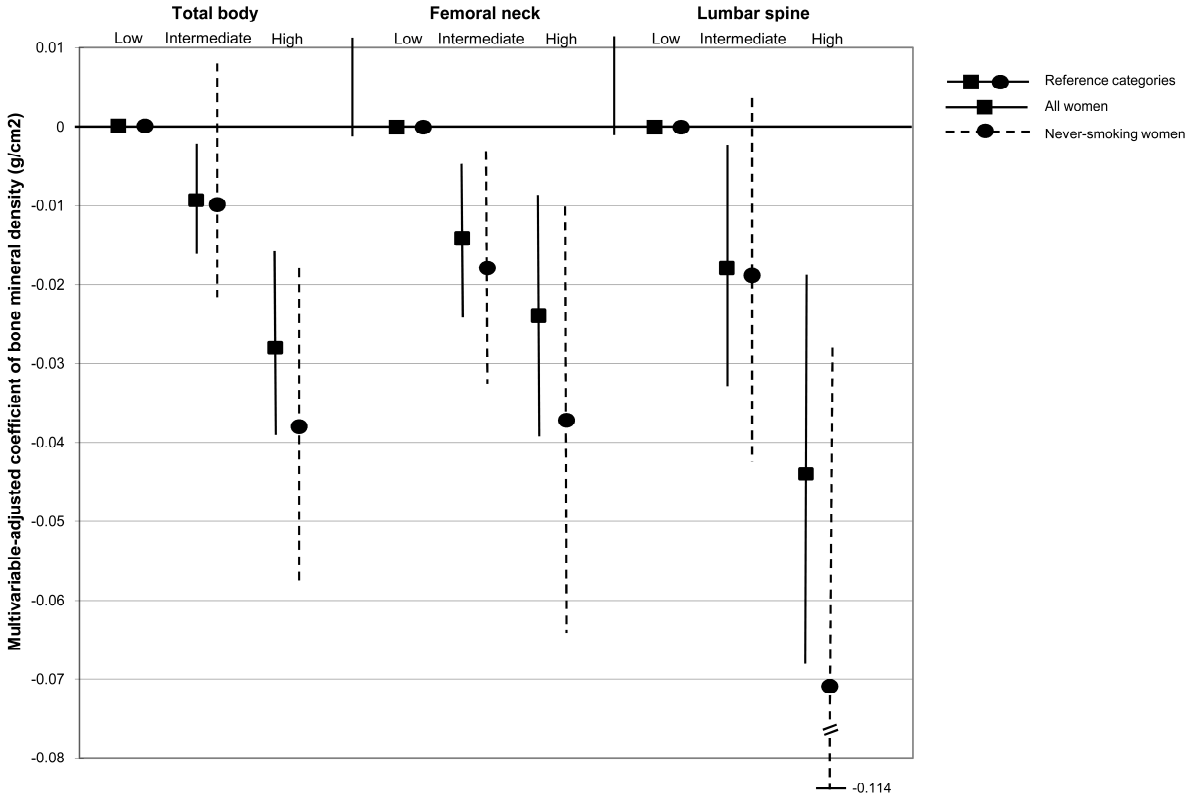

Figure 13. Multivariable-adjusted linear coefficient and 95% CI of BMD in relation to combined high dietary cadmium (≥13 µg/day), and high urinary cadmium (≥0.50 µg/g creatinine) as compared to the reference categories (–■–●–) of low dietary (<13 µg/day) and low urinary cadmium (<0.50 µg/g creatinine) among all women (–■–) and among never-smoking women (- ●-). The model was adjusted for age, education, body mass index, ever use of postmenopausal hormones, total physical activity, smoking status, alcohol intake, inflammatory joint diseases, and dietary intake of calcium, magnesium, iron, and fiber.

Table 4. Mean and median urinary and dietary cadmium in each category (<0.50 and ≥0.50 µg/g creatinine; <13 and ≥13 µg/day), respectively and in the combined analyses.

	Categories	**Mean**		**Median**	
Urinary cadmium (µg/g creatinine)	<0.50	0.28		0.27	
	≥0.50	0.65		0.60	
Dietary cadmium (µg/day)	<13	11		11	
	≥13	15		15	
		Urinary cadmium	Dietary cadmium	Urinary cadmium	Dietary cadmium
Low category	<0.50 and <13	0.28	11	0.27	11
Intermediate category	<0.50 and <13; ≥0.50 and ≥13	0.32	14	0.29	14
High category	≥0.50 and ≥13	0.66	15	0.60	14

A certain degree of misclassification of the women's long-term dietary cadmium exposure is not surprising as measurement error is inevitable due to it being based on self-report, dietary information in combination with the average cadmium concentration in each food item reported. By excluding women with implausible energy intake and by adjusting cadmium to the total caloric intake, under- or over reporting of habitual food intake is compensated for. However, as cadmium is not an intrinsic part of food the variation might be larger than for nutrients. Also the bioavailability of cadmium in food may differ substantially depending on both dietary factors and nutritional status, and that may lead to exposure misclassification in relation to the internal dose. For example, dietary cadmium may be less bioavailable in a high-fiber diet than in a low fiber-diet [40]. On the other hand, fermented fiber may increase e.g. the calcium uptake and thus increase the BMD [120, 121]. By adjusting our models by intake of dietary fiber, and calcium and magnesium some compensation of these variations seemed to be obtained. However, the most important factor for the bioavailability of cadmium in the diet seems to be the iron status of the individual. Low iron stores are associated with higher concentrations of cadmium in blood, urine and kidneys, as shown in several different populations [39, 40, 43, 45, 101, 122]. The likely explanation is the increased gastrointestinal uptake of cadmium *via* the apical divalent metal transporter 1 (DMT1) and the basolateral ferroportin 1 (FPN1) [123-126] in the enterocytes at iron deficiency. Low body iron stores and iron deficiency are common in premenopausal women due to menstrual losses and to pregnancy [127]. Unfortunately, for these Papers **(Paper II**), we did not have any information on the women's body iron

stores earlier in life. Although there is little evidence that the iron intake per se is related to the body iron stores, the association between dietary cadmium and bone was more pronounced after adjustment by dietary iron, supporting some confounding by this nutrient. Taking all these aggravating factors into consideration, it is indeed remarkable that an inverse association could be observed between the estimated dietary cadmium intake and bone.

More surprising, is that also the use of urinary cadmium alone led to underestimation of the risk (**Table 2, 6, 8; Figure 10, 14-17**), in turn indicating some exposure misclassification also by using urinary cadmium as a marker of long-term exposure. Cadmium in urine is considered to mainly reflect the kidney accumulation [6, 49] and thus the long-term integrated exposure from all sources, including non-dietary sources. Urinary cadmium concentrations increase with age but may decline due to aging [84]. We used an age cut-off <70 years in **Paper I and II** to avoid this exposure misclassification. However, we cannot exclude that the age (up to 69 years) has introduced some exposure misclassification, although there was no indication of this in the correlation between urinary cadmium and age (r_s=0.029, p=0.13; not shown in **Paper I** and **II**). Inter-individual variation in the toxicokinetics of cadmium and kidney physiology [38] as well as the method used to compensate for variation in urine dilution [128, 129] may affect how well urinary cadmium reflect the long-term exposure. In any case, by combining urinary cadmium with measurements of dietary cadmium intake, it seems as if the level of misclassification can be reduced.

5.2.2 Cadmium and osteoporosis

We also estimated the risk of osteoporosis in relation to cadmium exposure at sites particular susceptible to osteoporotic fractures (**Paper I and II**). Osteoporosis was defined as *T*-score <-2.5 which approximately corresponded to a cutoff in BMD of 0.70 g/cm^2 at the femoral neck, 0.67 g/cm^2 at the total hip, and 0.90 g/cm^2 at the lumbar spine. As expected, age was positively associated with the risk of osteoporosis at all sites (p<0.001); the mean age of women with osteoporosis was 65 years as compared to 64 years in those without osteoporosis.

The prevalence of osteoporosis was 8 and 10% at the femoral neck and lumbar spine, respectively and 15% when considering osteoporosis at either the femoral neck or lumbar spine (**Table 5**). This prevalence is only slightly lower than the 10% observed

at the femoral neck in 56 to 59 year old, the 14% observed in 60 to 64 year old and 20% in 65 to 69 year old women in Sweden [11], indicating that the women included in **Paper I** and **II** is fairly representative of Swedish women of similar age.

Women with osteoporosis had higher urinary cadmium concentrations and a slightly higher dietary cadmium intake than without osteoporosis (**Table 5**)**.**

Table 5. The prevalence of osteoporosis (OP) and the median concentrations of urinary cadmium (µg/g creatinine) and dietary cadmium (µg/day) in women with or without osteoporosis.

Site	*T*-score, <-2.5; n of women (%) with osteoporosis	Urinary cadmium OP *vs* no OP	Dietary cadmium[#] OP *vs* no OP	**Paper**
Femoral neck	216 (8.2%)	0.43 *vs* 0.34	-	**I**
Total hip	55 (2.1%)	0.43 *vs* 0.34	-	**I**
Lumbar spine	267 (10%)	0.38 *vs* 0.34	-	**I**
Hip or spine	400 (15%)	0.39 *vs* 0.34	12.9 *vs* 12.6	**I and II**

[#] Restricted to hip or spine to increase the statistical power.

In line with the results for BMD, we found significantly positive associations between urinary cadmium as a continuous variable, rescaled to 0.42 µg/g creatinine (equivalent to 2 SD) and risk of osteoporosis at the femoral neck, total hip, lumbar spine, and hip or spine (**Paper I**). The increased risk estimated per 2 SD-increment varied between 40% and 60% at the different sites. For categorized urinary cadmium, the highest exposure group (≥0.75µg/g creatinine) compared with the lowest (<0.50 µg/g creatinine) was associated with 2- to 3-fold increased risk of osteoporosis at all three sites. For femoral neck and hip or spine, also the second highest urinary cadmium exposure group (0.50 to 0.75 µg/g creatinine) was associated with higher risk of osteoporosis. To enable comparison with previous studies, we also calculated the risk of osteoporosis per µg/g creatinine in urinary cadmium (**Table 6**; data not shown in **Paper I,** except for total hip).

Table 6. Risk of osteoporosis and its 95% CI at the femoral neck, total hip, lumbar spine and hip or spine based on continuous urinary cadmium (per 1 µg/g creatinine increment) among all women and in the subgroup of never-smokers.

	Odds ratio[a] (95% CI)	
Site	All women	Never-smokers
Femoral neck	2.97 (1.61-5.52)	4.90 (1.57-15.5)
Total hip[b]	3.11 (1.23-7.88)	Numbers too small to perform analyses
Lumbar spine	2.27 (1.25-4.11)	2.04 (0.70-5.98)
Hip or spine	2.34 (1.39-3.95)	2.84 (1.12-7.19)

[a] Multivariable-adjusted for age, education, height, total fat mass, lean body mass, parity, use of postmenopausal hormones, ever use of corticosteroids, total physical activity, smoking status, alcohol intake, inflammatory joint diseases, kidney diseases, liver diseases, malabsorption.
[b] Model only adjusted for significant covariates (age, height, total fat mass, lean body mass and ever use of corticosteroids) because Hosmer-Lemeshow test indicated instability when all covariates were included.

In separate analyses among never-smokers the multivariable-adjusted OR for osteoporosis was 1.95 (95% CI 1.21–3.16) at the femoral neck and 1.55 (95% CI 1.05–2.29) at the hip or spine for every 0.42 µg/g of creatinine (=2 SD) increment in urinary cadmium (**Paper I)**. The highest exposure category of urinary cadmium (≥0.75 µg/g of creatinine) was associated with a 3- to 4-fold significantly increased risk of osteoporosis at the femoral neck, lumbar spine, and hip or spine. Thus, the association between urinary cadmium and risk of osteoporosis tended to be more pronounced among never-smoker than among all women. The reason for this difference is not known, but the women not classified as never-smokers were more heterogeneous both with respect to cadmium exposure and bone health. This group of women consists of both current and former smokers with a wide variety in the amount of cigarettes smoked. Smoking cessation is associated with a beneficial effect on bone and is often accompanied with an increase in body weight (also beneficial for bone), while the urinary cadmium concentrations may remain essentially the same. Altogether this may increase the total variation, attenuating the associations in all women. It can only be speculated that inhaled cadmium have a different toxic effect on bone than the cadmium absorbed *via* the diet. We found similar associations between dietary cadmium exposure and increased risk of osteoporosis at the hip or spine, as for urinary cadmium, although not as clear.

Per 10 µg/day-increment of dietary cadmium, we observed OR = 1.46 (95% CI: 0.97-2.20) (**Paper II**). By further adjustment for dietary calcium, magnesium, iron and fiber, we observed a significantly increased risk of osteoporosis, OR, 1.97 (95% CI: 1.12-3.48). The high dietary cadmium exposure category (≥13 µg/d = the median) as compared to the low (<13µg/d) was associated with a 32% significantly increased risk of osteoporosis (OR, 1.32 (95% CI, 1.02-1.71)) (**Figure 14B**). Among never-smoking women, a 34% increased risk was observed comparing high with low, but this was not statistically significant (OR, 1.34, 95% CI, 0-93-1.95) (**Figure 15B**). In the combined analysis, again the risk of osteoporosis was considerably higher than in the separate analyses, OR 2.49 (95% CI; 1.71-3.63) (**Figure 14C**) among all women and OR 2.65 (95% CI; 1.43-4.91) among never-smoking, respectively (**Figure 15C**). Results for urinary cadmium are given for comparison (**Figure 14A** and **15A**).

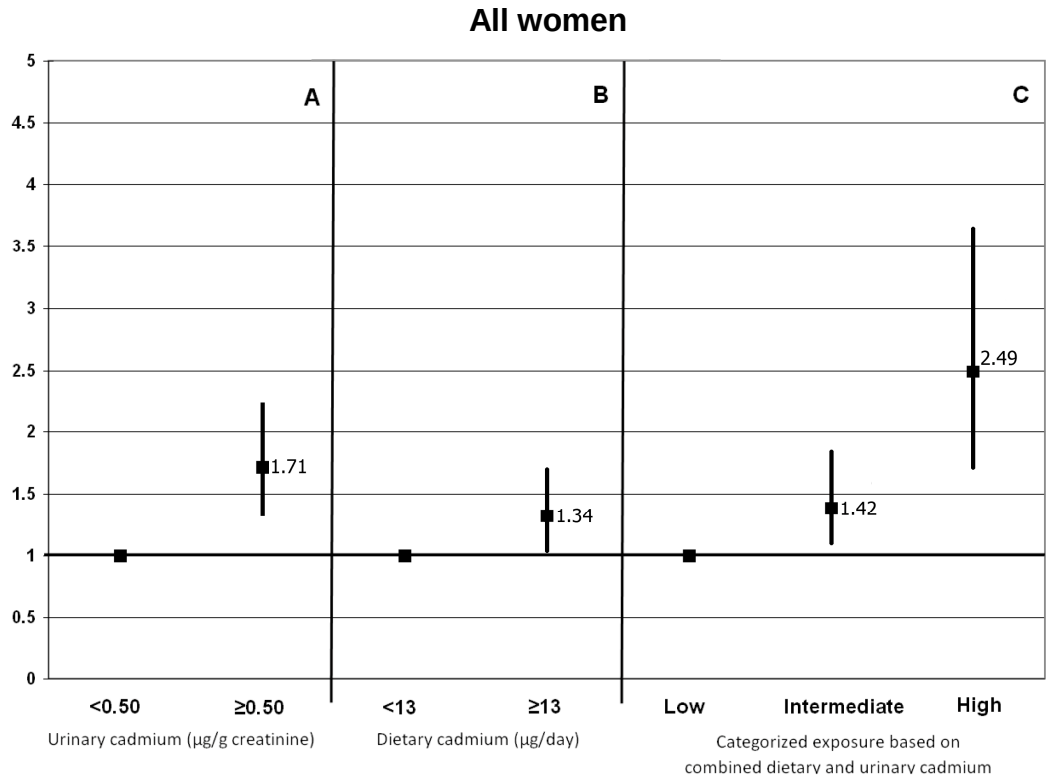

Figure 14. Multivariable-adjusted odds ratio and 95% CI of osteoporosis at the hip or spine among all women. Urinary cadmium (**A**) as categorized into below (low) or above (high) 0.50 µg/g creatinine; estimated dietary cadmium (**B**) as categorized into below (low) or above (high) the median, 13 µg/day and (**C**) with combined high urinary cadmium (≥0.50 µg/g creatinine) and dietary cadmium (≥13 µg/day), as compared to the reference category (<0.50 µg/g creatinine and <13 µg/day). For covariates; see **Paper I and II.**

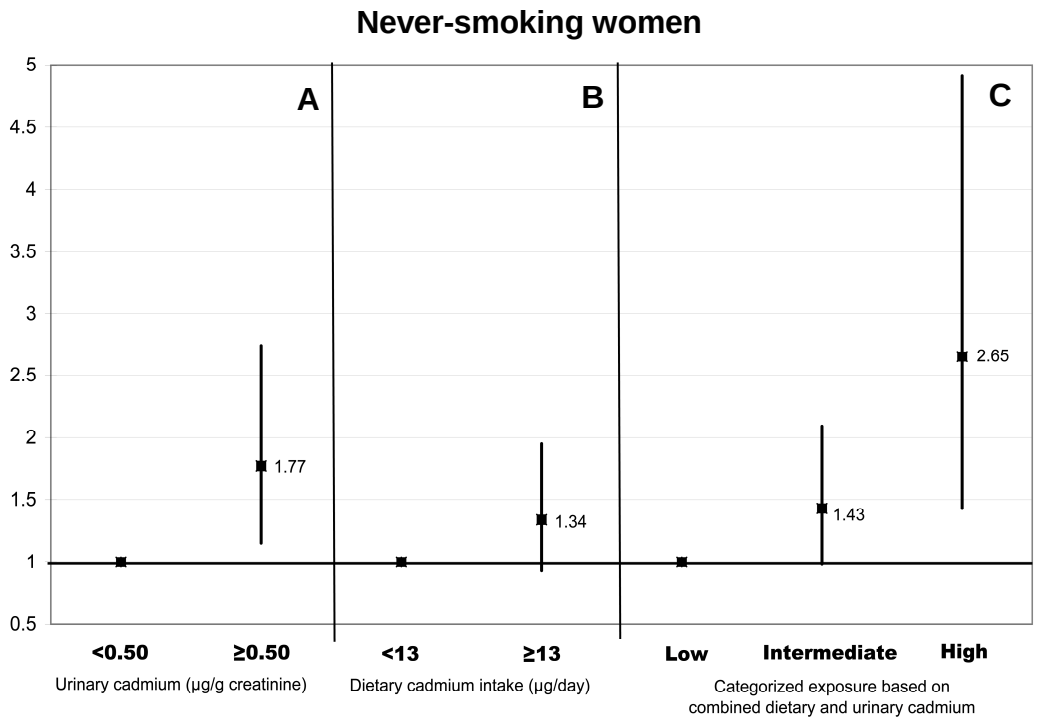

Figure 15. Multivariable-adjusted odds ratio and 95% CI of osteoporosis at the hip or spine among never-smoking women. Urinary cadmium (**A**) as categorized into below (low) or above (high) 0.50 µg/g creatinine; estimated dietary cadmium (**B**) as categorized into below (low) or above (high) the median, 13 µg/day and (**C**) with combined high urinary cadmium (≥0.50 µg/g creatinine) and dietary cadmium (≥13 µg/day), as compared to the reference category (<0.50 µg/g creatinine and <13 µg/day). For covariates; see **Paper I and II.**

As shown in **Table 4** (page 50), the more pronounced association observed after combining dietary with urinary cadmium (**Figure 13, 14C, and 15C**) was not due to unintentionally higher exposure in the high exposure category.

5.2.3 Cadmium and fractures

Besides BMD and the risk of osteoporosis, we also evaluated the risk of fractures (ascertained from 1997 to 2009) in relation to cadmium (**Paper I and II)**. The number of fractures is shown in **Table 7**. As expected, a fracture at the distal forearm was the most common fracture in women (**Paper I**). Given the limited number of fractures, we used only two categories of cadmium exposure. The concentration of cadmium in urine was slightly higher among women with fractures than without (**Table 7**).

Table 7. Total number and percent (%) of any first incident fracture, first osteoporotic fracture and the most common first fracture (distal forearm) among all women. The mean urinary cadmium concentration (µg/g creatinine) and the estimated dietary cadmium intake (µg/day) are compared between women with or without a fracture.

	Fractures, n (%)	**Urinary cadmium Fracture *vs* no fracture**	**Dietary cadmium Fracture *vs* no fracture**	**Paper**
Any first fracture	395 (15%)	0.43 *vs* 0.34	13.0 *vs*12.6	**I and II**
First osteoporotic fracture[a]	248 (9.2%)	0.43 *vs*.034	-	**I**
Most common first fracture; distal forearm	137 (5.1%)	0.38 *vs* 0.34	-	**I**

[a] Includes fractures of the hip, spine, distal forearm, proximal humerus, and pelvis.

In the analysis of any first incident fracture we observed per 0.42 µg/g creatinine (=2 SD) increment in urinary cadmium, a multivariable adjusted OR of 1.15 (95% CI, 0.92-1.43) among all women and a borderline statistically significant higher risk, OR 1.48 (95% CI, 1.00-2.17), among never-smokers. For categorized urinary cadmium, the higher risk of fractures among all women was also non-significant, OR, 1.16 (95% CI, 0.89-1.50) while a clearly statistically significantly higher risk was observed among never-smokers OR, 2.03 (95% CI, 1.33-3.09) comparing urinary cadmium ≥0.50 µg/g creatinine with lower levels). Similar results were observed in the analysis of any first osteoporotic fracture and first fracture of the distal forearm (**Paper I**).

To enable comparison with previous studies, we calculated the risk of fracture per 1 µg/g creatinine of urinary cadmium (**Table 8**; data not shown in **Paper I**).

Table 8. Multivariable-adjusted risks per 1 µg/g creatinine and its 95% CI of any first fracture, first osteoporotic fracture, and first distal forearm fracture, among all women and in the subgroup of never-smokers.

	Odds ratio[a] (95% CI)	
	All women	**Never-smokers**
Any first fracture	1.39 (0.82-2.34)	2.54 (1.00-6.33)
First osteoporotic fracture[b]	1.12 (0.57-2.15)	3.25 (1.15-9.29)
Most common first fracture; distal forearm	1.57 (0.70-3.64)	6.12 (1.80-21.1)

[a] Multivariable-adjusted for age, education, height, total fat mass, lean body mass, parity, use of postmenopausal hormones, ever use of corticosteroids, total physical activity, smoking status, alcohol intake, inflammatory joint diseases, kidney diseases, liver diseases, malabsorption.

It is well-known that low BMD explains a major part of the risk of fracture [11]. Thus, in additional analysis we explored whether the cadmium-related risk of fractures was mediated via lowered bone mass. Inclusion of total body BMD in the fracture models did, however, only partly remove the higher risks (**Table 5**; **Paper I**), indicating that cadmium exposure may result in other effects on bone that are not detected by DXA.

We also explored the risk of any first incident fracture in relation to dietary cadmium (**Paper II**). Per 10 µg/day-increment of dietary cadmium, we observed a non-statistically significant OR, 1.14 (95% CI: 0.76-1.71), which after further adjustment for the dietary intake of calcium, magnesium, iron, and fiber was OR = 1.44 (95% CI: 0.82-2.53) (**Paper II**). Women with a cadmium intake above the median, compared to values below median had an OR of 1.31 (95% CI: 1.02-1.69) and the corresponding OR among never-smokers was 1.54 (95% CI: 1.06-2.24) (**Figure 16B**). Results for urinary cadmium are given for comparison (**Figure 16A** and **17A**).

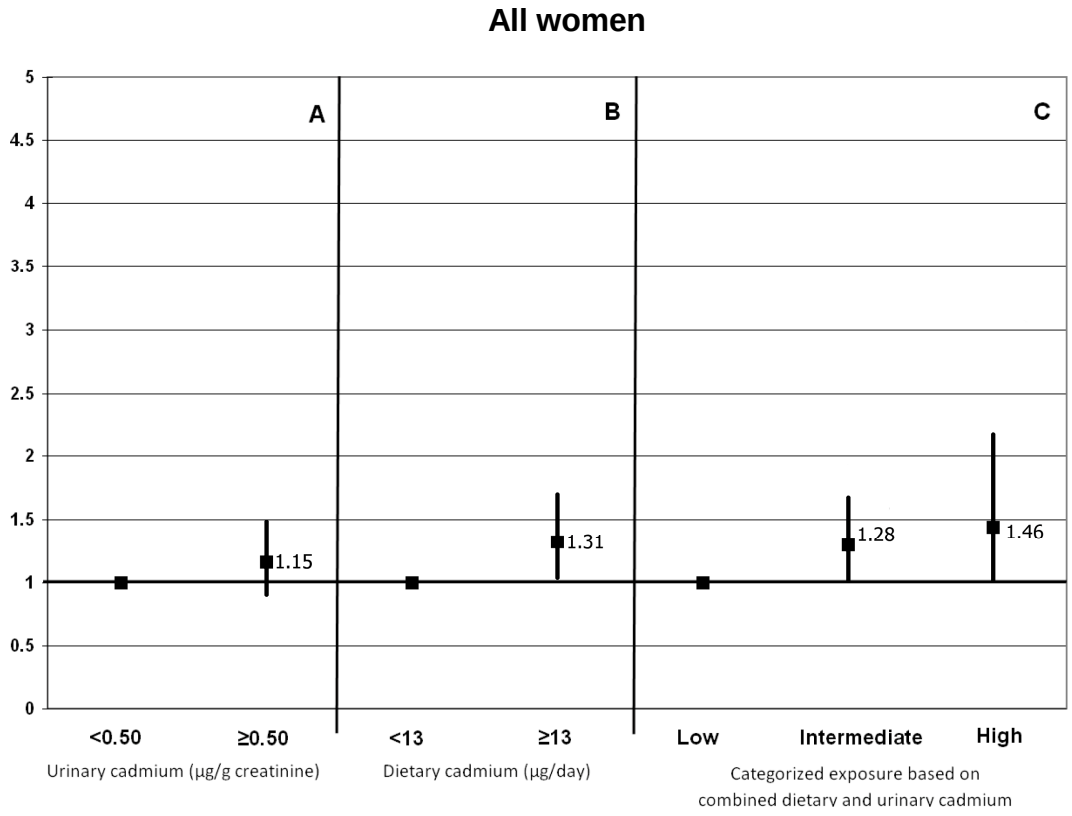

Figure 16. Multivariable-adjusted odds ratio and 95% CI of any first incident fracture among all women. Urinary cadmium (**A**) as categorized into below (low) or above (high) 0.50 µg/g creatinine; estimated dietary cadmium (**B**) as categorized into below (low) or above (high) the median, 13 µg/day and (**C**) with combined high urinary cadmium (≥0.50 µg/g creatinine) and dietary cadmium (≥13 µg/day), as compared to the reference category (<0.50 µg/g creatinine and <13 µg/day). For covariates; see **Paper I and II.**

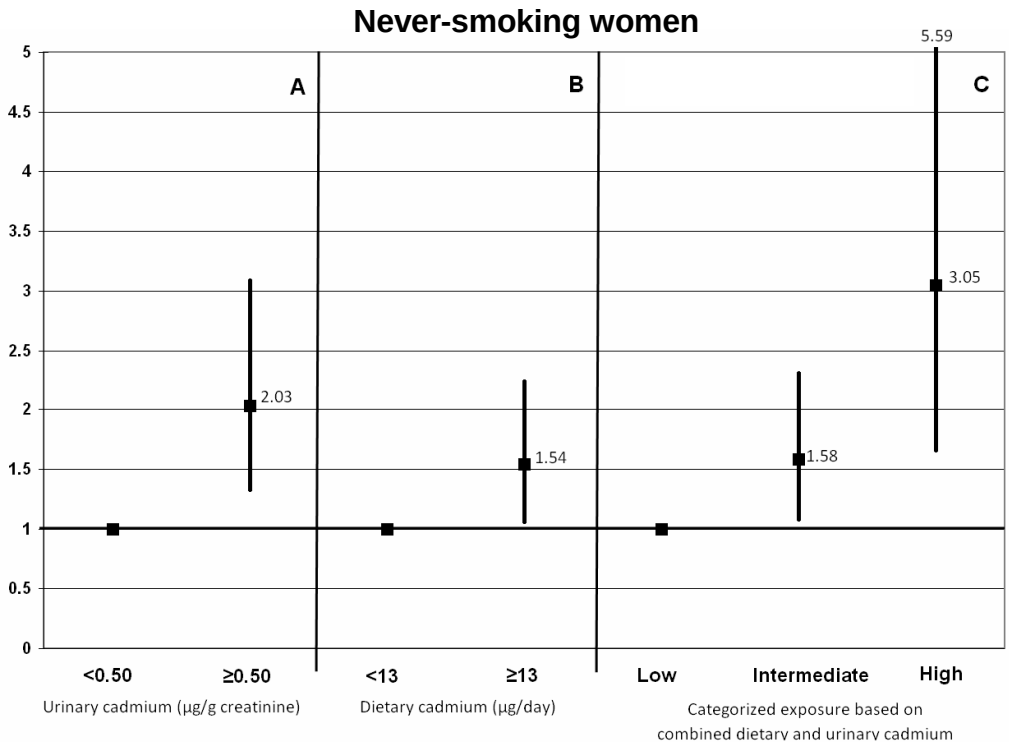

Figure 17. Multivariable-adjusted odds ratio and 95% CI of any first incident fracture among never-smoking women. Urinary cadmium (**A**) as categorized into below (low) or above (high) 0.50 µg/g creatinine; estimated dietary cadmium (**B**) as categorized into below (low) or above (high) the median, 13 µg/day and (**C**) with combined high urinary cadmium (≥0.50 µg/g creatinine) and dietary cadmium (≥13 µg/day), as compared to the reference category (<0.50 µg/g creatinine and <13 µg/day). For covariates; see **Paper I and II.**

As observed in the analyses for BMD and risk of osteoporosis, the combined analyses of high urinary and high dietary cadmium also revealed more pronounced associations with fractures. The corresponding results for risk of any first incident fracture were OR, 1.46 (95% CI: 1.00-2.15) among all women and OR, 3.05 (95% CI: 1.66-5.59) among never-smokers, respectively (**Figure 16C and 17C**).

Besides **Paper I and II,** only three studies have so far considered fracture incidence either using urinary cadmium or estimated dietary cadmium as the exposure marker. The two studies based on urinary cadmium were performed in both women and men in industrially contaminated areas in Belgium [55] and in Sweden [58]. The study based on dietary cadmium exposure (using the same database of cadmium content in food as in the present study) assessed fracture incidence in men in an area with no known industrial contamination [107]. In the study from Belgium, a two-fold increase of

urinary cadmium excretion was associated with a higher risk of any fracture in women; risk ratio (RR) 1.73 (95% CI, 1.16-2.57) but not in men. Cadmium was also analyzed in soil, leek and celery, sampled from the residence of the subjects, and was used as proxy of cadmium exposure. A similar risk estimate was observed as when using the urinary cadmium concentrations. The women in the study by Staessen and colleagues [55], had about twice the cadmium concentrations in urine as observed in the women in the present study (**Paper I**). Alfvén and colleagues [58] reported, per 1 nmol/mmol creatinine a hazard ratio (HR) of 1.18 (95% CI, 1.01–1.37) for forearm fracture in subjects over 50 years of age. Thomas et al [107] assessed the association between estimated dietary cadmium intake, comparing the highest with the lowest tertile. The risk of any first incident fractures was HR of 1.19 (95% CI, 1.06-1.34) as well as first incident hip fracture was HR 1.28 (95% CI, 0.97-1.69) in men from Sweden. In accordance with the findings in the present study (**Paper II**), the observed adverse associations between dietary cadmium intake and fractures were partly masked by dietary factors (i.e. fruits and vegetables).

The study by Thomas et al [107] is, besides **Paper I** and **II** the only to have evaluated the risk of fractures separately in never-smokers. In accordance with **Paper II,** the risk of hip fractures was more pronounced in never-smokers as compared to all men, HR, 1.70 (95% CI, 1.04-2.77) as compared to all men including smokers HR, 1.28 (95% CI, 0.97-1.69).

In conclusion, our results indicate that cadmium is a risk factor for osteoporosis and fractures. As the risk was generally more pronounced among never-smokers than among all women, it seems obvious that cadmium from the diet alone contributes to lower BMD, and increased risk of osteoporosis and fractures. The risk occurred at lower cadmium concentrations than previously observed, starting already at 0.50 µg/g creatinine, indicating larger concern than previously known. Because the high incidence of fractures is of major public health concern due to the reduced quality of life, reduced life expectancy and high costs for society associated with the disease, the results are of public health concern.

5.3 VITAMIN D, CADMIUM AND BONE

In **Paper III,** we assessed $1,25(OH)_2D$ in serum, selected on the basis of the lowest (n=45) and highest (n=40) urinary cadmium concentrations in WHILA. The median concentration of urinary cadmium in the low group was 0.25 µg/L (5-95th percentile; 0.14-0.39 µg/L), as compared to 1 µg/L (0.66-2.1 µgL) in the high cadmium group. The corresponding urinary cadmium concentration adjusted for creatinine was 0.36 µg/g creatinine and 1.1 µg/g creatinine, respectively. A similar exposure contrast was observed in blood. The concentration of $1,25(OH)_2D$ in all samples was 114 pmol/L (5-95th percentile; 67-171 pmol/L; n=85). The median concentration of 25(OH)D was 102 nmol/L (n=42), which is somewhat higher than previously observed in Swedish women and men [130-132]. The ratio between $1,25(OH)_2D$ and 25(OH)D was approximately 1:1000.

The women in the high-cadmium group had also significantly higher concentrations of markers of tubular damage and bone resorption, and a lower BMD and estimated lower glomerular filtration rate, as compared to the women in the low-cadmium group. This is in line with the results on cadmium-associated effects on both bone and kidney, previously reported in these women (n=820) [53, 61], and indicates a sufficient sample size. There was also a higher prevalence of women smoking in the high group, as compared to the low group. However, we found no statistically significant difference in serum $1,25(OH)_2D$ concentrations between the two urinary cadmium exposure groups. If anything, there was a tendency of a higher concentration of serum $1,25(OH)_2D$ in the high exposure group as compared to the low cadmium group (p=0.08). Furthermore, inclusion of 25(OH)D measurements in a subsample of the women, reduced the possibility that our null results were biased due to differences in vitamin D status.

In the final multivariable-adjusted model, only urinary calcium, besides either urinary or blood cadmium was included and, again, only urinary calcium, and not cadmium was significantly positively associated with $1,25(OH)_2D$. Thus, we interpret that the activation of $1,25(OH)_2D$, that mainly takes place in the kidneys was not affected by cadmium in these women. However, it cannot be completely ruled out that a compensatory slower degradation of $1,25(OH)_2D$ or increased activation of $1,25(OH)_2D$ in other tissues may have occurred [133] that could mask a possibly lower cadmium-induced activation in the kidney. In accordance with our results, some

experimental studies have observed no difference in $1,25(OH)_2D$ levels [134-136], or even higher $1,25(OH)_2D$ levels in rats treated with cadmium for 90 days [134], as compared to non-exposed controls. Sacco-Gibson and colleagues did not find any change in $1,25(OH)_2D$ levels or in renal dysfunction in dogs exposed to cadmium for seven months, as compared to non-exposed controls, despite increased bone resorption (skeletal ^{45}Ca release) [136]. In contrast, two experimental studies indicate lower levels of $1,25(OH)_2D$ in serum and kidney in female rats exposed to cadmium at a level relevant to humans [114, 115]. Previous human studies are inconclusive [71, 73, 74].

The results from **Paper III** may suggest that there is no general involvement of cadmium-induced kidney damage in the effects on bone. Thus, these effects on could be parallel events, or that the tubular dysfunction increases the excretion of calcium and phosphate, as shown in experimental studies [114, 136] and some [56, 62] but not all [53] human studies, where in turn increased losses of calcium may cause bone loss . However, cadmium may have a direct toxic effect on bone, possibly through accelerated differentiation of osteoclasts, causing higher bone resorption [61, 62, 70, 136-141]. To compensate increased release of calcium from bone to the circulation, excess calcium is excreted in urine

In conclusion, these results indicate that, even though there were clear associations between cadmium and bone- and kidney effect markers, the activation of $1,25(OH)_2D$ seemed not to be affected. These findings add some light on the mechanism of cadmium-induced effects on bone.

5.4 RETINOL, CADMIUM AND BONE

In **Paper IV**, we assessed serum retinol concentrations in 606 women. The obtained concentrations (median 1.9 µmol/L, ranging from 0.97 to 4.3 µmol/L), were mainly within what is considered the normal range of 1-3 µmol/L [27], and similar to that observed in women in USA and Europe [142-145] but somewhat lower than those obtained in Swedish men [32, 146].

The main characteristics of the women in relation to tertiles of serum retinol are shown in **Paper IV**. Women in the highest tertile were less often classified as never-smokers and being postmenopausal and had lower concentrations of bALP and osteocalcin as

compared to those in lowest tertile, while no difference was observed for BMD. No significant correlation was observed between serum retinol and urinary cadmium concentrations (p=0.87).

In the multivariable-adjusted analysis serum retinol was significantly inversely associated with bALP and osteocalcin ($p \leq 0.04$), and was close to significantly positively associated with BMD (p=0.08) and PTH (p=0.07) but not with DPD (p=0.25). Urinary cadmium on the other hand, was still after including serum retinol in the models, inversely associated with BMD and PTH (p=0.01 for both) and with DPD ($p<0.001$), but not with bALP or osteocalcin (p=0.19 and p=0.40, respectively). Altogether, this indicates that retinol and cadmium may have contrasting effects on bone.

We observed a tendency of a positive association between serum retinol and BMD. Null or weak associations have previously been observed for serum retinol or retinyl esters in relation to BMD or fracture risk [143-150]. Some studies have found retinol to be associated with higher BMD, or have shown that women with osteoporosis have lower retinol concentrations. In **Paper IV**, we found no indication of a U-shaped or non-linear relationship between vitamin A and the bone outcomes, as indicated in some previous studies [34, 35]. Although, the serum retinol concentrations in the present study were in the similar range as that observed in the two studies from Opotowsky and colleagues and Promislow and colleagues, although we only observed few women that had concentrations below 1 µmol/L or above 3 µmol/L, which may have compromised the possibility to observe any U-shaped relationship.

We further evaluated the combined effect of serum retinol and urinary cadmium by categorizing serum retinol and cadmium into 2 groups: below (low) or above (high) the median (< and ≥1.9 µmol/L for serum retinol and < and ≥0.66 nmol/mmol creatinine for urinary cadmium). Women with serum retinol below the median in combination with cadmium above the median had lower BMD (p=0.016); as compared to those with combined elevated serum retinol and lower urinary cadmium (**Figure 18A**). We also stratified the analyses by smoking status (never/ever), as smoking is an additional source of cadmium exposure and adversely associated with BMD [5, 151], and may negatively affect the concentration of vitamin A [143, 152]. Among never-smoking

women, higher serum retinol (≥1.9 µmol/L) was associated with higher BMD independent of urinary cadmium levels (**Figure 18B**).

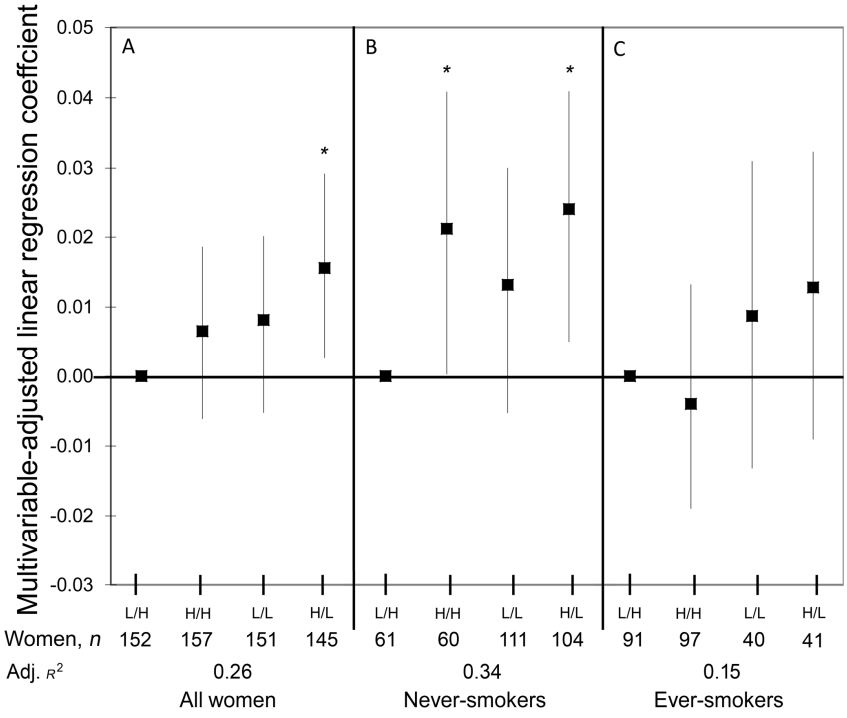

Figure 18. Multivariable-adjusted linear regression coefficient and 95% CI of BMD in relation to combined serum retinol (categorized into below (low, L) or above (high, H) the median; 1.9 µmol/L) and urinary cadmium (categorized into below (low, L) or above (high, H) the median; 0.66 nmol/mmol creatinine) among all women (A), never-smokers (B) and ever-smokers (C). Low serum retinol and high urinary cadmium constitute the reference category. Model was adjusted for age, BMI, menopausal status, season and physical activity. *Different from the reference category, $p<0.05$.

Among ever-smoking women, no significant association was observed (**Figure 18C**), although there was a tendency of a higher BMD in women having high retinol and low cadmium.

In conclusion, our findings may suggest the negative effect of cadmium on bone may be counteracted by vitamin A.

5.5 ADDITIONAL METHODOLOGICAL CONSIDERATIONS

5.5.1 Study design

Papers I-IV are based on cross-sectional data with the exception of the analysis of risk of fractures (ascertained from 1997 to 2009). In **Paper I**, fractures were ascertained both retrospectively and prospectively in relation to the urinary cadmium measured in 2004-2008. In **Paper II,** fractures were ascertained prospectively in relation to dietary cadmium intake, estimated via the FFQ in 1997. It should be noted, however, that the study population in **Paper I** and **II** was defined by those who had provided urine samples in 2004 to 2008.

The cross-sectional design is often used when the purpose is to describe a population or to estimate the prevalence, but not the incidence, of the outcome of interest at a certain time point. This design is also common in studies using biomarkers of exposure and/or effects. Nearly all studies assessing associations between cadmium and bone are of cross-sectional design. However, since both the exposure and the outcome are measured at the same time, it will hamper the inference with respect to causality. It could be argued that the use of urinary cadmium is somewhat different since this marker reflects the long-term kidney accumulation over decades. In a cross-sectional study, it is important that the participating subjects are representative of the population and a high participation rate is required to be able to generalize the findings to the general population. The participation rate was relatively high in the studies.

With a prospective design the exposure is measured before the outcome is observed, which is an advantage as e.g. the diet is not affected by the disease (**Paper II**). In a retrospective design the outcome is first observed and then the exposure is estimated, although it seems unlikely that the urinary cadmium concentrations were affected by fracture status (**Paper I**).

5.5.2 Selection bias

Selection bias is a systematic error that may occur when the participating subject differ from those not participating. The bias may be introduced when the procedure choosing the participants differ, or when other factors influence the participation (Rothman, 1998). High participation rate is therefore important. The participation rate in the

present studies varied between 60 and 70%, which generally is considered relatively high,. In **Paper I** and **II**, we were only able to include women who were recruited between 2004 and 2008 (no urine samples were collected before 2004 and recruitment continued until September 2009). The women recruited during 2009**,** did not differ in e.g. BMI from those included in this study, indicating that the slightly shorter recruitment period did not affect the representativness of women. For the whole sub-cohort, however, we cannot exclude that women with limited mobility due to e.g. fractures were less likely to participate and that those who participated were healthier than those who did not participate. Nevertheless, about a similar prevalence of osteoporosis was observed in the studies as compared to the prevalence estimated in Swedish women of similar age [11].

A systematic error may be introduced in prospective studies if there are differences in completeness of the follow-up between the exposed and unexposed. By computerized linkage to the National Patient Registry and regional fracture registries, we considered the follow-up of fractures virtually complete, minimizing the possibility that our results were biased by differential follow-up.

5.5.3 Information bias

Information bias occurs when measurements or classification of exposure or disease do not correctly measure what they are suppose to measure i.e. they are not valid. These errors may be introduced by the participants, by the instrument (laboratory or questionnaire) or by the observer. The main type of misclassification in this thesis which may affect the interpretation of exposure-disease is non-differential. This misclassification refers to errors in the measurement of exposure that are unrelated to the disease, or errors in classification of disease that are unrelated to the exposure. Thus, if this misclassification does not differ between the exposed or unexposed or with or without the outcome, this would mainly give rise to underestimation of the strength of the association.

Random and systematic errors

There is always a possibility of random errors in quantitative research. If random errors are lacking, then the precision is high. Precision depends mostly upon sample size but also on the quality of the data. Although our study is one of the largest using biomarkers of cadmium exposure (i.e. adequately powered) and at the same time

having a high analytical accuracy, the number of cases was limited in some analysis resulting in wide confidence intervals. Systematic errors in turn are not dependent upon sample size or chance, but rather a methodological error that may occur when selecting the study participants.

Misclassification of exposure

To minimize exposure misclassification, all analyses were performed with a high analytical accuracy (see Methods). Both urinary and dietary cadmium may be prone to misclassification. In **Paper I and II**, we choose a cut-off <70 years in order to decrease the risk of distortion of the urinary cadmium concentration that may occur in older age [84]. In **Paper III and IV** all women were below 64 year of age. In any case, this most likely attenuates the observed associations.

We performed several sensitivity analyses with regard to urinary cadmium in order to test the robustness of the results. First we excluded subjects that had extreme creatinine concentrations (outside 0.3 and 3.0 g/L, n=65), which had only marginal effects on the estimates. Very low creatinine concentrations (a sign of low muscle mass) leads to higher creatinine-adjusted urinary cadmium concentration, while a very high creatinine concentration give rise to lower adjusted urinary cadmium concentrations. In additional analysis, the creatinine concentration was included as covariate in the multivariable-adjusted models (together with creatinine-adjusted cadmium) to account for some additional explanation of creatinine; again, this had no effect on the estimates. Secondly, we adjusted urinary cadmium to mean urinary density instead of creatinine in order to minimize a possible effect of muscle mass; also this approach had marginal effect on the estimates.

There is always a risk that the results are dependent on how the exposure categories are chosen. In **Paper I**, several attempts were made to explore possible effects of the categorization. The exposure (i.e. urinary cadmium) was included either as continuous variable, categorized in predetermined exposure categories or into tertiles. The results showed that the analyses were very robust. With regard to the estimated dietary cadmium exposure, misclassification is inevitable with this kind of methods, due to error in self-reports and due to normal within-person variation in intake over time [153, 154] (see page 49-50 for more details). Since **Paper II** is a prospective study and any measurement error that would result in misclassification is unrelated to the outcome,

this would produce non-differential misclassification. Thus, this would most likely lead to an underestimation of the true relationship.

Misclassification of outcome

Misclassification of outcome may also occur. The use of a phantom and the high precision in the BMD measurements ensured a high validity. The consistency in the results between the different skeletal sites measured; support a low degree of misclassification of the outcome. Since fractures were obtained from registries (**Paper I and II**) with nearly 100% case ascertainment this misclassification is most likely minimal. Also the 1,25(OH)D concentrations in serum may be prone to misclassification. However, the control samples were within the recommended values and the CV was low, indicating that this misclassification should be rather low.

5.5.4 Confounding

In **Paper I-IV**, potential confounders were chosen based on whether they were associated with both the exposure and outcome, changed the estimate more than 10% in the model, or were generally accepted risk- or protective factors. Age and tobacco smoking are examples of very important confounders, clearly associated with both exposure and with the effect. A major advantage with the studies in this thesis is that we were able to control for several potential confounders. Confounders may also be misclassified and several strategies were undertaken to decrease the level of misclassification: For instance in order to be classified as being a never-smoker, the women had to report that they had never smoked in at least two separate questionnaires. On the other hand, combining former and current smokers into one category (instead of using two separate categories or pack-years) may have led to residual confounding, but was done in order not to over load the statistical models and because the main focus was on never-smokers. On the other hand, as the distinction between former smoking and current smoking is not fully evident, misclassification may persist even if smokers were categorized into three groups.

5.5.5 Generalizability

The participants in the SMC and WHILA study are from the general population of Central and Southern Sweden. The relatively high participation rate indicates that the results are generalizable to the middle-aged female Swedish population.

6 CONCLUSIONS

- Cadmium in urine, a marker of long-term exposure, was associated with lower BMD and increased risk of osteoporosis and fractures in Swedish women. The associations were independent of tobacco smoking, indicating that cadmium from the diet alone contributes to the risk.

- Dietary cadmium exposure, estimated *via* a food frequency questionnaire, was associated with lower BMD and increased risk of osteoporosis and fractures. These associations were partly masked by dietary factors important for bone health and cadmium bioavailability.

- Women with combined high dietary and urinary cadmium had lower BMD and a more pronounced increased risk of osteoporosis and fractures, indicating an underestimation of the risk in the separate analysis of urinary or dietary cadmium.

- We found no support for cadmium-associated bone effects being mediated via lower concentrations of $1,25(OH)_2D$.

- Serum retinol seemed to counteract some of the negative effects of cadmium on bone.

These findings are of high public health relevance as cadmium exposure is prevalent and osteoporotic fractures substantially contribute to the total burden of disease. Moreover, the exposure occurs mainly *via* valuable foods such as whole-grain cereals, vegetables and potatoes. The associations occurred at lower exposures than previously observed, providing support for revision of the existing health risk assessment.

7 FUTURE RESEARCH

The present research has increased the knowledge on the association between cadmium exposure and bone effects in the general population. Future research should include:

- Experimental studies are needed to increase the understanding on the mechanism(s) of the effects on bone.

- A meta-analysis should be performed to summarise the evidence from all studies and to evaluate the dose-response relationship between urinary cadmium and bone. This is necessary to be able to set a “reference point” for bone effects that may be used in future health risk assessments.

8 POPULÄRVETENSKAPLIG SAMMANFATTNING

Kadmium är ett metalliskt grundämne naturligt förekommande jordskorpan. Tillförseln av kadmium till åkermark sker via nedfall från luften (från tex metalltillverkning, förbränning av fossila bränslen, sopförbränning etc.) och genom gödsling (handelsgödsel och rötslam). Kadmium tas lätt tas upp i växter via rotsystemet vilket gör att kosten är den huvudsakliga exponeringskällan för de flesta människor. Höga halter av kadmium återfinns i födoämnen som skaldjur, inälvsmat och vissa fröer. Det största bidraget till exponering sker dock via våra viktigaste baslivsmedel såsom spannmålsprodukter, potatis och grönsaker och rotfrukter. Rökare exponeras ytterligare för kadmium via tobaksrök. Kadmium ansamlas i kroppen, främst i njuren. Det är sedan länge känt att kadmium orsakar skador på njurarna. Vid massiv exponering har man även sett att kadmium orsakar skelettskador med frakturer som följd. Några få studier talar för att även en mycket lägre exponering dvs den exponering som förekommer i den allmänna befolkningen kan påverka benhälsan och möjligen öka risken för benskörhet. Sambanden är dock ofullständigt klarlagda.

Benskörhet kallas “den tysta epidemin” då den utvecklas långsamt och ger inga symptom innan den första frakturen uppstår. Benskörhetsfrakturer utgör ett stort folkhälsoproblem som orsakar mycket lidande och höga kostnader för samhället, vilket belyser behovet av prevention. Varannan kvinna och var fjärde man förväntas att drabbas av en benskörhetsfraktur under sin livstid. Varje år beräknas 70 000 benskörhetsfraktur inträffa i Sverige till en kostnad över 5.6 miljarder, enbart inkluderat sjukvårdskostnader. Det beräknas att endast hälften av de som drabbas av en höftfraktur återvänder till ett självständigt liv. Dödligheten efter en höftfraktur ligger mellan 10-15%. Det är stora geografiska skillnader i insjuknandet i höftfrakturer, sannolikenheten är mer än sjufaldigt högre att drabbas i norra Europa, speciellt i Sverige och Norge, än i övriga Europa.

Syftet med denna avhandling var att undersöka effekterna av långsiktig låggradig kadmiumexponering på benhälsan, och utröna om dessa effekter uppkommer via minskad aktivering av vitamin D i njuren. Ett annat syfte var att belysa möjlig kombinerade effekt av kadmium och vitamin A på ben. Två befolkningsbaserade studier användes som består av kvinnor, 54 till 69 år: Den Svenska

Mammografikohorten (SMC) i Uppsala och Kvinnors Hälsa i Lundabygden (WHILA). Kvinnornas kadmiumexponering bestämdes genom analys av kadmiumhalten i urin, vilket reflekterar den kroniska exponeringen, och genom att uppskatta kadmiumintaget via en kostenkät. Kvinnornas bentäthet mättes i flera olika delar i kroppen med hjälp av röntgenteknik (DXA). Dessutom har information om frakturer inhämtats *via* frakturregister. Vitamin D och vitamin A analyserades i serum.

Resultaten visade tydliga negativa samband mellan kadmium och bentäthet i helkroppen, lårbenshalsen, höften och i ländryggen, dvs med ökande kadmiumexponering så minskar bentätheten. Vi fann också samband mellan kadmiumexponering och ökad risk att drabbas av benskörhet och frakturer. Samtliga samband var oberoende av tobaksrökning. Vidare fann vi inget belägg för att kadmium stör vitamin D-aktiveringen i njuren. Vitamin A verkade delvis motverka kadmiums negativa effekt på ben. Anmärkningsvärt var att sambanden kunde påvisas vid låg exponering dvs den exponering som återfinns i den allmänna befolkningen

Sammanfattningsvis så bidrar denna avhandling med viktig information om kadmiums negativa effekt på ben med både ökad risk för benskörhet och frakturer. Dessa fynd är av hög relevans eftersom alla är exponerade för kadmium, den huvudsakliga exponeringen sker *via* våra viktigaste livsmedel och det finns inga tecken på att exponeringen minskar. Dessutom utgör benskörhetsfrakturer ett stort folkhälsoproblem.

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