Cadmium, kidney and bone

Maria Wallin

Department of Occupational and Environmental Medicine
Institute of Medicine
Sahlgrenska Academy at University of Gothenburg, Sweden

UNIVERSITY OF GOTHENBURG

Gothenburg 2015

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maria.wallin@amm.gu.se

ISBN (printed) 978-91-628-9577-8
ISBN (e-publication) 978-91-628-9578-5
Electronic version available at: http://hdl.handle.net/2077/39550

Printed in Gothenburg, Sweden 2015
Printed by Ineko AB

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ABSTRACT

Toxic heavy metals, such as cadmium, mercury and lead, occur in the environment both naturally and as contaminants due to agricultural and industrial activities. The aims of this thesis were to examine the levels of these metals in the kidney in the general population and the association with sources of exposure, and to study the effects of low-level cadmium exposure on kidney and bone. The first three studies were cross-sectional and were conducted on 109 living kidney donors. In the first study, the concentrations of cadmium, mercury and lead in kidney cortex, and the impact of different exposure sources and background factors, were examined. Kidney cadmium levels were relatively low (median 12.9 µg/g wet weight), and increased with age, smoking, and in women with low iron stores. Kidney mercury levels were associated with the number of amalgam surfaces, but not with fish consumption. Kidney lead levels were very low, and not related to any of the background factors. In the second study, the relationships between kidney cadmium, urinary calcium and bone mineral density were investigated. A positive association was found between kidney cadmium and calcium excretion in women, but not in men. Negative correlations were found between kidney cadmium and bone mineral density, but the associations were not significant after adjustment for covariates. In the third study, the relation between kidney cadmium and kidney function was explored, and significant positive associations were found with the excretion of alpha-1-microglobulin, but there was no association with glomerular filtration rate. The fourth study was both cross-sectional and prospective, and was conducted on 936 elderly men. Negative associations were found between urinary cadmium and bone mineral density, and those with high urinary cadmium had an increased risk of incident non-vertebral osteoporosis fractures. In conclusion, the results of this thesis indicate effects of cadmium on kidney and bone also at the low levels found in the general population in Sweden. This provides further support for the importance of reducing the spread of cadmium in the environment.

Keywords: cadmium, kidney function, bone, urinary calcium, fracture

ISBN (printed): 978-91-628-9577-8 **ISBN (e-publ.):** 978-91-628-9578-5

SAMMANFATTNING PÅ SVENSKA

Giftiga tungmetaller som kadmium, kvicksilver och bly finns i vår omgivningsmiljö, både naturligt och till följd av utsläpp från jordbruk och industrier. Målet med den här avhandlingen var att undersöka halterna av dessa metaller i njuren hos allmänbefolkningen och sambandet med olika källor till exponering, samt att studera effekten av exponering för låga halter av kadmium på njurar och skelett.

De första tre studierna i avhandlingen var tvärsnittsstudier och utfördes på 109 levande njurdonatorer i Sverige. I den första studien undersöktes koncentrationerna av kadmium, kvicksilver och bly i njurbarken och inverkan av olika exponeringskällor och bakgrundsfaktorer. Halten av kadmium i njuren var relativt låg (medianvärde 12,9 μg/g våtvikt), men ökade med åldern, antalet rökta cigaretter under livet och hos kvinnor med låga järndepåer. Halten av kvicksilver i njuren ökade med antalet amalgamytor på tänderna, men påverkades inte av fiskintaget. Halten av bly i njuren var mycket låg och uppvisade inget samband med de undersökta bakgrundsfaktorerna. I den andra studien undersöktes sambanden mellan njurkadmium, kalcium i urinen och bentätheten. Vi fann ett positivt samband mellan njurkadmium och kalciumutsöndringen hos kvinnor, men inte hos män. Det fanns dock inget säkert samband mellan njurkadmium och bentäthet. I den tredje studien undersökte vi sambandet mellan njurkadmium och njurfunktion och fann ett positivt samband med utsöndringen av det lågmolekylära proteinet alfa-1-mikroglobulin i urinen, men inget samband med den glomerulära filtrationshastigheten. I den fjärde studien, som både var en tvärsnittsstudie och en prospektiv studie, undersöktes 936 äldre män från Göteborg. Vi fann negativa samband mellan urinkadmium och bentäthet, och de med högt urinkadmium hade högre risk att senare få en benskörhetsrelaterad fraktur i höft, underarm, överarm eller bäcken.

Sammanfattningsvis tyder resultaten i avhandlingen på att kadmium påverkar njurar och skelett även vid de låga nivåer som förekommer i allmänbefolkningen i Sverige. Detta ger ytterligare stöd åt vikten av att minska spridningen av kadmium i omgivningsmiljön.

LIST OF PAPERS

This thesis is based on the following studies, referred to in the text by their Roman numerals.

I. Barregard L, Fabricius-Lagging E, Lundh T, Mölne J, Wallin M, Olausson M, Modigh C, Sallsten G. Cadmium, mercury, and lead in kidney cortex of living kidney donors: Impact of different exposure sources. *Environmental Research. 2010 Jan;110(1):47-54.*

II. Wallin M, Sallsten G, Fabricius-Lagging E, Öhrn C, Lundh T, Barregard L. Kidney cadmium levels and associations with urinary calcium and bone mineral density: a cross-sectional study in Sweden. *Environmental Health. 2013 Mar 7;12:22.*

III. Wallin M, Sallsten G, Lundh T, Barregard L. Low-level cadmium exposure and effects on kidney function. *Occupational and Environmental Medicine. 2014 Dec;71(12):848-54.*

IV. Wallin M, Barregard L, Sallsten G, Lundh T, Karlsson MK, Lorentzon M, Ohlsson C, Mellström D. Low-level cadmium exposure is associated with decreased bone mineral density and increased risk of incident fractures in elderly men: the MrOS Sweden study. *Submitted manuscript.*

CONTENT

ABBREVIATIONS

A1M	Alpha-1-microglobulin, protein HC
B-Cd	Cadmium in blood
B2M	Beta-2-microglobulin
BMD	Bone mineral density
BMI	Body mass index
Cd	Cadmium
CI	Confidence interval
Crea	Normalized for creatinine, for example A1MCrea (mg/g creatinine)
eGFR	Estimated glomerular filtration rate
GFR	Glomerular filtration rate
GM	Geometric mean
Hg	Mercury
HR	Hazard ratio
ICP-MS	Inductively coupled plasma mass spectrometry
K-Cd	Cadmium concentration in kidney cortex
kDa	Kilodalton
KIM-1	Kidney injury molecule 1
LMW	Low molecular weight
LOD	Limit of detection
mGFR	Measured glomerular filtration rate
µg/gC	Microgram per gram creatinine

ON	Overnight sample
OR	Odds ratio
Pb	Lead
r_p	Pearson's correlation coefficient
r_s	Spearman's correlation coefficient
RBP	Retinol-binding protein
SD	Standard deviation
SHBG	Sex hormone-binding globulin
U-Cd	Cadmium in urine
ww	Wet weight

Conversion factors

1 µg Cd ~ 8.9 nmol Cd

1 µg Hg ~ 5 nmol Hg

1 g creatinine ~ 8.8 mmol creatinine

1 µg Cd/g creatinine ~ 1 nmol Cd/mmol creatinine

1 INTRODUCTION

1.1 Toxic heavy metals – cadmium, mercury, and lead

Cadmium, mercury and lead are heavy metals that occur naturally in the environment, but since the industrial revolution, human activities have contributed significantly to their dispersal (1-3). All three metals are well-known to be toxic to human health, with both acute and chronic effects. Much of the knowledge about health effects originally derive from disasters with extreme exposure levels or from studies on occupationally exposed workers. However, in this thesis the emphasis is on low-level exposure in the general population.

As all three metals are nephrotoxic and accumulate in the kidney, the concentrations in kidney cortex are of particular interest. However, kidney biopsies from living humans are seldom available because of the risks associated with the procedure, and therefore most of the previous knowledge on metal concentrations in the kidney comes from autopsy studies (4-20).

Cadmium

Since the focus of this thesis lies on cadmium, this metal will be described in more detail in the later sections (subchapter 1.2-1.4).

Mercury

Apart from the portion that occurs naturally, mercury is present in the environment for example as a result of mining, emissions from industries, and incineration of waste and fossil fuels (21). Due to its toxicity, the Swedish Government decided to ban the use of mercury and mercury-containing articles in 2009. There are however some exceptions to the ban, for example mercury in light sources (22).

Mercury occurs in three primary forms; elemental mercury, and inorganic and organic mercury compounds (3). Elemental mercury (Hg^0, also called liquid silver, quicksilver, colloidal mercury or hydrargum) exists either as a liquid or as a gas (mercury vapor). The general population is exposed to elemental mercury mainly via inhalation, and dental amalgam fillings can be a significant source of exposure (3, 23). About 80% of inhaled elemental mercury is retained, while less than 0.01% is absorbed in the gastrointestinal tract after ingestion. In the human body, elemental mercury rapidly

undergoes oxidation to its inorganic divalent form, Hg^{2+}, which is thought to be the toxic species as elemental mercury cannot react with tissue ligands (3, 24).

Inorganic mercury compounds have been used in ethnic or folk medical practices for different purposes, and are also used in cosmetics such as skin-lightning creams (3, 23). Unlike elemental mercury, oral ingestion is the main route of exposure for inorganic mercury salts. The intestinal absorption of inorganic mercury is higher than for elemental mercury, about 10% (3). Inorganic mercury can also be absorbed through the skin.

Inorganic mercury is mainly accumulated in the kidney, followed by the liver (23). Elemental mercury and inorganic mercury compounds are eliminated from the body mainly by excretion in urine, but also in feces (3, 24). Since mercury in urine (U-Hg) is directly derived from mercury previously deposited in the renal tissue, U-Hg is probably a good indicator of the kidney burden of mercury, and maybe also a rough indicator of the total body burden (24). The half time for U-Hg after cessation of long-term occupational exposure has been found to be about 2-3 months (25, 26).

Adverse health effects in humans are induced by all forms of mercury (21). Exposure to high doses of elemental mercury can cause damage to most human organs, but the central nervous system is the most sensitive part of the body. The main organs affected by acute poisoning with inorganic mercury compounds are the intestine and the kidneys (23, 24). Health effects following chronic exposure to elemental or inorganic mercury mainly include neurological and behavioral symptoms or disorders, which in severe cases may be irreversible, but kidney damage has also been reported (3). Possible health effects of exposure to mercury from dental amalgam have long been a concern, both for patients and dental personnel. Some studies have shown subtle effects mainly on cognitive function, but many large studies show no such effects (3, 23).

Methyl mercury (MeHg) is the most studied form of organic mercury compounds affecting human health, and the main source of low-level exposure in the general population is fish (23). Inorganic mercury (both Hg^0 and Hg^{2+}) can be transformed to MeHg by microorganisms in water, and then bioaccumulated in larger organisms, such as fish (24, 27). Almost 100% of ingested MeHg is absorbed from the gastrointestinal tract, and about 80% is absorbed after inhalation of MeHg vapor (23). In addition, absorption can occur through intact skin. MeHg is accumulated in the body, especially in the brain, and may cause neurological symptoms and disorders.

MeHg can also be transferred to the fetus through the placenta, and cause disturbed motor and mental development of the child. MeHg is mainly excreted via bile into feces. In addition, MeHg is demethylated in the body to inorganic mercury (27). The absorbed amount of MeHg is reflected by the concentrations in blood, hair, or toenails (27). As only small amounts of MeHg are excreted in urine, U-Hg mainly reflects exposure to inorganic mercury (24). However, almost no inorganic mercury is accumulated in hair, so this is considered to be a biomarker of MeHg exposure (24).

Lead

Lead is also released to the environment both from natural sources, such as volcanic activity, and from anthropogenic activities, for example mining, combustion of municipal waste and fossil fuels, and metal processing. In 2003, the use of lead batteries accounted for 78% of the reported global consumption of lead (1). Humans are mainly exposed via inhalation of air and dust, intake of food and beverages, and also, especially in children, ingestion of dust and soil. In countries where it is still allowed to use lead in petrol, this can be an important source of exposure through inhalation. In Sweden, lead in petrol has been banned since 1995, and the most important exposure sources for humans are food and beverages (22). For example kidney, liver, and seafood may contain relatively high levels of lead (22). However, the European Food Safety Authority (EFSA) has calculated that the largest contributors to lead exposure from foodstuff in Europe are vegetables, nuts, pulses and cereals (28). Lead is also present in tobacco plants, and smoking, as well as exposure to environmental tobacco smoke, has been found to be associated with somewhat higher levels of lead in blood (28, 29).

Lead is very toxic also in low doses, and can cause neurological, renal, gastrointestinal, cardiovascular, reproductive and hematological damage (1). It accumulates in the body, especially in bone (>90% of the body burden), but also in the liver and kidneys (1, 21). The most commonly used biomarker of exposure is lead in blood (B-Pb), with an initial half-life of about 1 month (21). In epidemiological studies, bone lead has often been used as a biomarker as it better reflects long-term exposure, having a half-life of about 10-30 years (28). Lead is excreted mainly via urine and feces, but lead in urine is seldom used as a biomarker of exposure due to large variations in the association between the concentrations of lead in urine and blood (21). Alike MeHg, lead passes through the placenta to the fetus (1). The critical effect, which may occur at low levels of exposure, is considered to be neurodevelopmental disturbances in children. In several studies, adverse health effects in children have been reported at B-Pb levels at 10 µg/dl, or

even below this level, but no threshold for the effects is known (1, 30). In recent years, many countries have reported decreasing B-Pb levels in the population correlating to the decreased use of lead in petrol.

1.2 Cadmium

Exposure

Inorganic cadmium occurs naturally following for example weathering of rocks and volcanic eruptions, but human activities have contributed to increasing levels of cadmium in air, water and soil, and also in many living organisms (2, 31). For example, cadmium can be released following processing or combustion of raw materials containing cadmium impurities, such as minerals and fossil fuels, as well as recycled materials (2). Agricultural soil can be contaminated by air deposition of cadmium, and by the spread of cadmium-containing sewage sludge and phosphate fertilizers (31). Cadmium in the soil is then taken up by growing plants, which are eaten by animals and humans (31).

Cadmium is mainly produced as a by-product of the mining and refining of zinc, and to some extent also of lead and copper (2, 32). In its elemental form, cadmium is a silver-white and soft metal (32). It has some special qualities that make it useful in a number of areas, including low melting temperature, high ductility and conductivity, and very good resistance to corrosion (32). Refined cadmium is mainly used in nickel-cadmium batteries, but also in for example pigments in plastics and ceramics, plating and coating, alloys and stabilizers for plastics (32). In Sweden, the use of cadmium is tightly regulated, but cadmium is still allowed for example as a pigment in colors used by artists (22).

In the general population, food and cigarette smoking are the main sources of cadmium. In non-smokers, about 90% of the cadmium exposure is derived from food and less than 10% from ambient air and drinking water (2, 31). Most food contains cadmium, but agricultural crops usually account for the largest part of the intake. In Sweden, potatoes and wheat flour contribute to 40-50% of the dietary exposure (33). The average intake of cadmium in Europe and North America is about 10-20 µg/day (21).

Occupational exposure occurs mainly via inhalation, but ingestion of contaminated dust and food can occur (32). Those particularly at risk of exposure are workers in cadmium production and refineries, industries producing nickel-cadmium batteries, cadmium pigments and cadmium

alloys, mechanical plating companies, zinc smelters and polyvinylchloride compounding industries (32).

Uptake, distribution and elimination of cadmium

After inhalation, 10-50% of cadmium is absorbed in the lungs, while only 3-5% of dietary cadmium is absorbed in the gut (31, 34). However, those with low iron stores are particularly vulnerable to cadmium exposure as they have a higher absorption rate (2, 34-36). The reason for this is probably that iron deficiency causes an up-regulation of a common receptor in the intestine (36, 37). In Sweden, 10-40% of fertile women have been reported to have S-ferritin <12 µg/L, indicating very low iron stores (34). Cadmium absorption is also increased in those with a low intake of calcium and zinc (21).

After absorption to the blood, cadmium is largely bound to high molecular weight (HMW) proteins like albumin, and transported via the bloodstream to the liver, where it forms a complex with the protein metallothionein (Cd-MT) (34, 38, 39). In addition, cadmium can bind directly to the small amounts of MT circulating in blood, and probably also to thiol-containing amino acids and peptides (38, 39). Most of the cadmium in blood is however found in the red blood cells (34). The Cd-MT complex is further transported by the blood to other parts of the body, and the initial half-life of cadmium in blood is two to three months (34). In the kidney, Cd-MT is filtered in the glomeruli and then reabsorbed in the proximal tubules, where toxic effects occur following the release of cadmium ions (21, 34). Cadmium is then accumulated mainly in the kidney cortex, where it has a long biological half-life of 10-30 years, but also in the liver, muscle, and bone (21, 31, 34, 40). About 0.01-0.02% of the body burden of cadmium is excreted each day via urine and feces (21, 40).

Biomarkers of cadmium exposure

The most commonly used biomarkers of cadmium exposure are cadmium in blood (B-Cd) and cadmium in urine (U-Cd). B-Cd is used as a marker of both recent and cumulative exposure, while U-Cd mainly reflects cumulative exposure (32). However, as cadmium accumulates in the kidneys, the concentration in the kidney (K-Cd) is often considered to be the most reliable biomarker of cumulative cadmium exposure. About 1/3-1/2 of the total body burden of cadmium is accumulated in the kidneys, with a 1.25 times higher concentration in kidney cortex than in the total kidney (21). K-Cd increases with age up to 50 to 60 years of age, when it usually begins to decline (34).

As B-Cd responds quickly to changes in exposure, it can be used for monitoring of workers with potential occupational exposure, reflecting current exposure (34). However, when the exposure is low B-Cd is to a large extent dependent on the cumulative exposure, and can be used as an estimate of the level in the kidney, or the body burden of cadmium (34). As a result of this, B-Cd usually increases with age (34). Cadmium in urine is often the best readily available biomarker of cumulative exposure to cadmium, and U-Cd is proportional to the concentration of in the kidney (34, 40). However, if cadmium causes renal tubular damage, Cd-MT reabsorption decreases and U-Cd increases, which in the end can lead to lower K-Cd and finally also lower U-Cd, thus no longer reflecting the cumulative exposure (34).

Previously, the ratio between U-Cd (µg/g creatinine) and K-Cd (µg/g) was assumed to be about 1:20 (34). In a recent study, the U-Cd/K-Cd ratio was however found to be about 1:60 at a K-Cd level of 25 µg/g, and the relation was found to be nonlinear, with decreased excretion rate at higher K-Cd in individuals with normal GFR (40).

Health effects of cadmium

Cadmium is a non-essential metal for humans, and toxic to many organs, particularly kidney and bone. Negative health effects were first reported in 1858, as workers exposed to a cadmium-containing polishing powder developed acute symptoms from the gastrointestinal system, and also delayed symptoms from the airways (41). In the 1940s, acute gastrointestinal symptoms were reported after oral intake of contaminated food and beverages, and cases of osteomalacia, emphysema and proteinuria were observed after occupational exposure (41, 42). In the 1950s the Itai-itai disease in Japan, characterized by osteomalacia, osteoporosis and fractures, but also renal dysfunction, was identified as a disease caused by cadmium in highly contaminated rice, as a result of the irrigation of rice fields with polluted water (41).

The kidney has long been considered the critical target for cadmium toxicity, with an increased excretion of proteins in urine as the first sign of renal damage (2). However, in recent years, increasing attention has been paid to the effects of low-level cadmium exposure on bone, focusing on the risk for osteoporosis and fractures at levels found in the general population (43). This will be more deeply discussed in the following sections (subchapter 1.3-1.4).

Cadmium and cadmium compounds have also been classified as carcinogenic to humans (Group 1) by the International Agency for Research

on Cancer (32). The type of cancer mainly associated with cadmium is lung cancer, but associations have also been found with cancer in the prostate and kidney (32, 44). In a few studies statistical associations have also been found between cadmium and cancer in the endometrium, bladder and breast (31, 32, 45, 46). In Sweden, two large population-based cohort studies have shown significant associations between dietary cadmium and cancer in the prostate, endometrium, and breast (44-46). Furthermore, cadmium has recently been associated with cardiovascular diseases, but it is too early to say whether this is a causal relationship (47-49).

1.3 Kidney function and renal effects of cadmium

Ever since the 1950s, when the cause of the Itai-itai disease in Japan was identified, it has been well-known that high doses of cadmium can cause kidney damage in humans. However, the effects of low-level exposure are not as clear.

The kidneys have an important role in maintaining homeostasis in the human body (i.e. maintaining the constancy of the internal environment) by controlling the concentration of waste products of metabolism, the osmolality, the volume, the acid-base status and the ionic composition of the extra-cellular fluid, and indirectly also affecting the intra-cellular fluid (50). Humans normally have two kidneys, situated on each side of the vertebral column, each about 12 cm long and with a weight of 150 g. There is a slit on the medial part of the kidney called the hilus, through which run the renal artery, the renal vein, lymph vessels, the renal nerve, and the dilated upper end of the ureter, called the renal pelvis. The kidney is divided into two regions; the cortex, which is the darker outer part, and the medulla, which is the paler inner part.

Each kidney contains about 1-1.5 millions of nephrons, which are the functional units of the kidney (50). The cortex contains the part of the nephron called Bowman's capsule and the proximal and distal tubules (Figure 1). The medulla contains the loop of Henle and the collecting duct. Bowman's capsule encircles the glomerulus, which is a knot of blood capillaries. As blood flows through the glomerulus it is filtrated, passing into Bowman's capsule, forming an ultrafiltrate almost free of proteins. The molecular weight cut-off for the glomerular filtration is about 70 kDa, and only small amounts of large plasma proteins like albumin (with a molecular weight of 68 kDa) can pass (50). Molecules with a weight below 7 kDa can

pass freely through the filter into the nephron. The ultrafiltrate passes from Bowman's capsule into the proximal tubule, and further down to the medulla where the proximal tubule becomes the descending loop of Henle. The ascending loop of Henle reaches into the cortex and becomes the distal tubule. The distal tubules of the nephrons then drain into the collecting ducts.

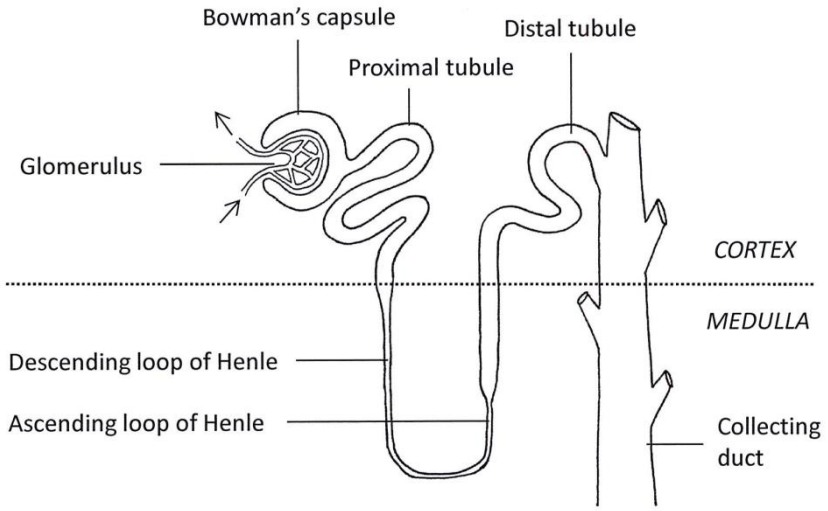

Figure 1. The nephron.

In the proximal tubules, reabsorption of a number of solutes takes place, including sodium, chloride, potassium, urea, glucose, amino acids, phosphate, calcium, and water (50). Hydrogen ions are secreted into the proximal tubules, as part of the regulation of the acid base balance. In addition, most proteins that are filtered in the glomeruli are normally reabsorbed in the proximal tubules, after binding to specific sites. After this receptor mediated endocytosis, the proteins are cleaved into amino acids in the cell's lysosomes. In the thick ascending limb of the loop of Henle, and in the early part of the distal tubule, the tubular fluid is diluted as a result of the active transport of sodium into the medullary interstitium. In the collecting ducts, urine is concentrated by osmotic abstraction of water (50).

The glomerular filtration rate (GFR) is considered to be the best overall measure of kidney function, and the gold standard method is to measure GFR (mGFR) using urinary or plasma clearance of exogenous filtration markers, for example iohexol or inulin (51). Ideally, this marker is a substance that passes the glomerular filter easily, and is not absorbed, secreted or metabolized by the kidney, but completely secreted in urine (50). A simpler method is to estimate GFR (eGFR) using equations based on endogenous filtration markers such as serum cystatin C or creatinine (51). However, as other factors than kidney function, for example muscle mass, might affect the endogenous marker, eGFR sometimes differs greatly from mGFR (51).

GFR is about 180 L/day (120-125 mL/min per 1.73 m^2 body surface area) in young adults (50). GFR declines gradually with increasing age, especially after the age of 50 when the decrease is about 10 mL/min/1.73 m^2 per ten year-period (52). GFR $\geq$90 mL/min/1.73 m^2 is usually considered normal, whereas 60-89 mL/min/1.73 m^2 is considered to be mildly decreased GFR (52, 53). At GFR levels below 60 mL/min/1.73 m^2, the risk for complications of kidney disease increases (53). According to the National Kidney Foundation in the United States, chronic kidney disease is defined as either kidney damage or decreased GFR (<60 mL/min/1.73 m^2) for at least 3 months, and persistent proteinuria is the principal marker of kidney damage (53, 54).

Cadmium toxicity typically affects the proximal tubules in the kidney, but impaired glomerular filtration and renal failure have also been reported (55). Increased urinary excretion of low molecular weight (LMW) proteins, as a result of decreased reabsorption in the proximal tubules, is often the first sign of renal tubular damage (34). Many different biomarkers have been used in studies of cadmium-induced renal effects. The most common renal biomarkers used for screening of populations at risk are the LMW proteins beta-2-microglobulin (B2M), retinol-binding protein (RBP), and alpha-1-microglobulin (A1M, also called protein HC) (56). U-B2M and U-RBP have been considered to be sensitive biomarkers of renal tubular damage, whereas U-A1M is less specific, and might be increased also in glomerular disease (56). U-B2M has been most commonly used, but RBP and A1M are more stable in urine (56). Other biomarkers of renal dysfunction are creatinine and cystatin C in serum, and albumin (Alb), N-acetyl-beta-D-glucosaminidase (NAG), and kidney injury molecule 1 (KIM-1) in urine. S-cystatin C is a LMW protein that reflects glomerular function, and has a high sensitivity in mild and moderate kidney damage (57, 58). Albumin in urine can be elevated as a result of both glomerular and tubular dysfunction, and is often

used in screening for early kidney damage in patients with hypertension or diabetes (57). NAG is a lysosomal enzyme predominantly present in the proximal tubules, and can be elevated in urine as a result of tubular damage, but also after increased lysosomal activity or glomerular dysfunction (57, 58). KIM-1 is a tubular transmembrane protein, and increased excretion in urine mainly reflects tubulointerstitial damage (57).

In the past 10-15 years, a number of studies have shown effects on the urinary excretion of proteins at very low levels of cadmium exposure (59). In 2009, the European Food Safety Authority (EFSA) established a tolerable weekly intake (TWI) of 2.5 µg cadmium/kg body weight, based on kidney effects as the critical endpoint (increased excretion of U-B2M) (55). According to EFSA's risk assessment, long-term dietary cadmium intake at this level (TWI) would result in a U-Cd below the critical concentration in 95% of the population by age 50 (55). Increased excretion of LMW proteins in urine has been thought to occur at U-Cd >4 µg/g creatinine, but after adjustment for inter-individual variation, the critical concentration in urine was set to 1.0 µg Cd/g creatinine (55). However, in most previous studies on cadmium-induced renal effects in humans, U-Cd was used as the only biomarker of cadmium exposure and/or cadmium body burden (59). One potential problem with the use of U-Cd as a biomarker at low-level exposure is co-excretion of cadmium and proteins in urine due to physiological factors (60-62). It has recently been suggested that non-renal effects, such as bone effects and cancer, should be considered to be the critical effects of cadmium in humans (43).

1.4 Osteoporosis, fractures, and bone effects of cadmium

Osteoporosis is a common bone disease characterized by decreased bone mass and disrupted bone architecture (63). The definition of osteoporosis according to WHO is a bone mineral density (BMD) of at least 2.5 standard deviations below the mean BMD of a young female adult (T-score ≤ -2.5), usually measured by dual energy X-ray absorptiometry (DXA), preferably at the femoral neck (64, 65). The disease is often not diagnosed until the patient gets a fragility fracture, which is the most serious consequence of the disease, resulting in suffering, disability, and increased mortality for the patients, as well as extensive costs to society (63). Common sites for major osteoporotic fractures are the hip, the distal forearm, the shoulder (proximal humerus), and the spine. It has been estimated that about 107,000 incident (new) fragility fractures occurred in Sweden in 2010 (63). Age-standardized

hip fracture incidence in Sweden, and also in the other Scandinavian countries, is among the highest in the world (66).

Osteoporosis has long been seen mainly as a disease affecting post-menopausal women, but there is now a growing awareness that osteoporosis is a major health problem for men as well (65). Most previous research has been conducted on women, but more studies are now conducted in order to identify risk factors in men. Some of the clinical risk factors for osteoporotic fractures in men that have been identified so far are high age, low body mass index, high alcohol consumption, current smoking, chronic use of corticosteroids, and a history of falls, prior fractures, stroke, diabetes and hypogonadism (67). Sex steroids are important regulators of bone metabolism, and low levels of serum estradiol have been associated with low BMD and increased fracture risk both in men and women (68-70). In men, also low levels of testosterone and high levels of sex hormone-binding globulin (SHBG) have been associated with increased fracture risk, but the results are not as consistent as for estradiol (68). In addition, parental hip fracture, low physical activity, and chronic kidney disease have been associated with an increased fracture risk in both sexes (71-73).

As previously mentioned, high-level exposure to cadmium has been associated with osteomalacia, osteoporosis and fractures for a long time, especially since the eruption of the Itai-itai disease in Japan, but the effect of low-level exposure has not been known. However, in recent years a number of studies have pointed towards an association between low cadmium exposure, at levels found in the general population, and an increased risk of osteoporosis and fragility fractures (43, 74-84). This is supported by experimental studies on rats with long-term dietary exposure to cadmium, resulting in decreased bone mineral density and increased bone fragility (85).

The mechanism(s) behind the effects of cadmium on bone are still uncertain. A number of theories have however been presented, such as an influence of cadmium on parathyroid hormone (PTH) or on vitamin D activation in the kidney, interference with calcium absorption in the gut, impaired reabsorption of calcium in the renal tubules, or a direct effect on bone (Figure 2) (21, 34, 59, 86-90). Experimental studies in bone culture systems indicate a direct effect of cadmium on bone cells, causing both decreased bone formation and increased bone resorption (85).

1.5 Calcium metabolism

An average man with a weight of 70 kg contains about 1 kg calcium, mainly within bone which consists largely of complex salts of calcium and phosphate (50). Calcium is however also present in extracellular fluids, and as it affects the excitability of nerves and muscles, it is important for the body to regulate the calcium concentration very accurately. Calcium in extracellular fluids either comes from intestinal absorption of dietary calcium, or from bone, and can be lost via urine or included in bone tissue (Figure 2). The excretion of calcium in urine each day is usually equal to the net absorption in the gut.

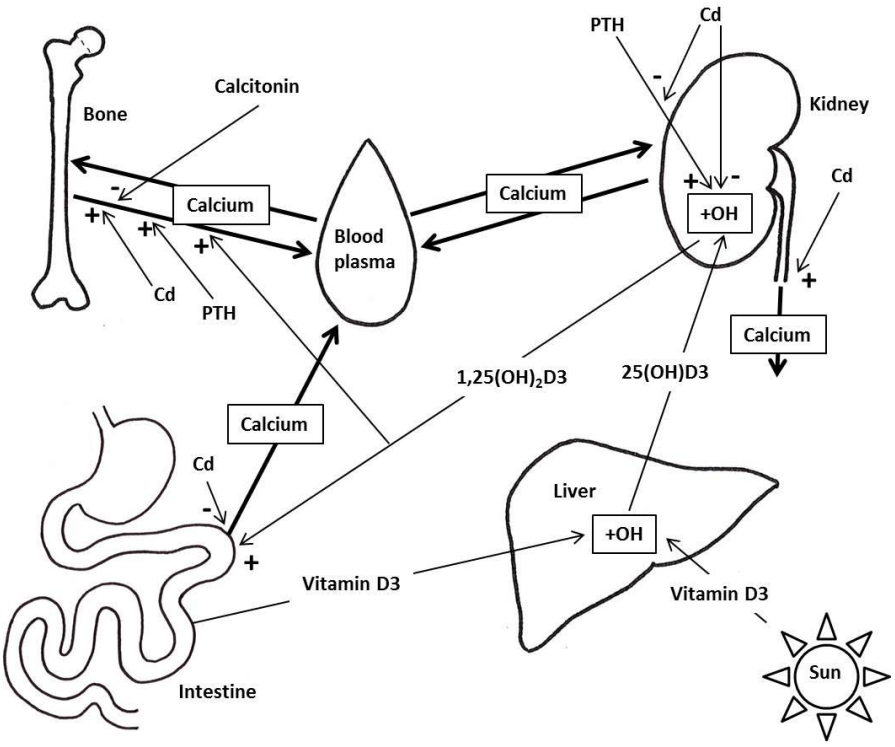

Figure 2. Proposed effects of cadmium on calcium and vitamin D metabolism. Modified from Nordberg et al. 2015 (21).

In the plasma, about 50% of calcium is present as ionized calcium, and about 50% is bound to other molecules, mainly proteins such as albumin. Unlike bound calcium, most of the ionized calcium is filtered in the glomeruli, but more than 95% is normally reabsorbed. In the proximal tubule and the ascending loop of Henle, calcium reabsorption is mainly passive, but there is also some active absorption. However, the main part of the physiological regulation of the reabsorption of calcium takes place in the cortical thick ascending limb and the distal tubule of the nephron (50).

Parathyroid hormone (PTH) and vitamin D are the main regulators of calcium (and phosphate) homeostasis (50). PTH secretion from the parathyroid glands is stimulated if the plasma concentration of ionized calcium is decreased, and diminished if the plasma concentration is increased. PTH acts mainly on bone, stimulating bone resorption, but also stimulates vitamin D activation in the kidney (Figure 2). Active vitamin D (calcitriol or $1,25(OH)_2D3$) is a steroid that is produced from precursors by a number of metabolic steps in the liver and the kidneys. The vitamin D precursors are either derived from the diet or formed in the skin after exposure to sunlight. Vitamin D increases plasma calcium mainly by enhancing the absorption of calcium and phosphate from the gut, but also by increasing the resorption from bone, and probably also by increasing the reabsorption of calcium in the nephron. The hormone calcitonin has the opposite effect, decreasing extracellular calcium concentration by reducing the release from bone tissue (50). Cadmium has been proposed to affect the urinary excretion of calcium either by decreasing the reabsorption of calcium in the kidney, or by increasing the release of calcium from bone, as mentioned in subchapter 1.4.

2 AIMS OF THE THESIS

The overall aims of this thesis were to increase knowledge on the levels of toxic heavy metals in the general population and associations with different sources of exposure, and to study the effects of low-level cadmium exposure on kidney and bone.

The specific aims were:

- to examine the concentrations of cadmium, lead and mercury in kidney cortex biopsies from living kidney donors in Sweden (Paper I)

- to assess the impact of different exposure sources and background factors on the levels of cadmium, lead and mercury in kidney cortex biopsies (Paper I)

- to explore the relation between kidney cadmium levels in kidney donors and:

    1. urinary calcium (Paper II)
    2. bone mineral density (Paper II)
    3. kidney function (Paper III)

- to study the associations between urinary cadmium and bone mineral density, as well as the risk of future fractures, in a cohort of elderly men in Gothenburg (Paper IV)

3 MATERIALS AND METHODS

3.1 Paper I, II and III – the TINA study

3.1.1 Study population and sampling

Paper I, II and III are based on data from a cross-sectional study on living kidney donors (the TINA study) conducted in Gothenburg, Sweden between January 1999 and June 2002, and between April 2004 and February 2005. During these two periods, 188 transplantations with living kidney donors were performed at the Department of Transplantation and Liver Surgery at Sahlgrenska University Hospital. Twenty-one of these donors were not eligible for the study; for example some of the donors were not able to take part in the study protocol, living abroad or the recipient was a child. The remaining 167 kidney donors were invited to take part in the study. As fifteen did not want to participate, 152 donors (81% of all donors, 91% of those invited) were included in the study after informed consent. The median age was 50 years (range 24-70 years). 87 of the 152 donors were women (57%) and 65 were men (43%). The study was approved by the Ethics Committee at the University of Gothenburg.

All donors were examined with routine blood and urine tests, kidney function tests and radiology less than one year before transplantation, according to a standard protocol. One or two days before transplantation, the donors were admitted to the hospital and underwent further routine examinations as well as tests according to the study protocol. A timed overnight urine sample was taken the morning after admission to the hospital. In addition, most donors provided a separate timed 24-hour urine sample. They also underwent a physical examination and an interview about their medical history and medication, and answered a questionnaire on occupational history, smoking habits, and diet. The total number of dental amalgam surfaces and the number of occlusal amalgam surfaces were counted for each donor.

During transplantation, a biopsy was taken from the donor's kidney cortex in 126 of 152 donors, as part of the routine. In 109 of those 126 donors (87%), part of the biopsy from the kidney was transferred into a pre-weighed acid-washed glass tube and frozen for later analysis of heavy metals. Those 109 donors formed the final study population in paper I, II and III. The median age in this group was similar to that of the whole group, 51 years (range 24-

70 years), as was the proportion of women and men (55% and 45%, respectively). There were 38% never-smokers, 38% former smokers and 25% current smokers.

3.1.2 Data from the questionnaire

Occupational history

All donors were asked to list their former and current occupations, and what year they began and quit in each one. They were also asked if they had worked with cadmium, lead or mercury (yes/no/do not know). Occupational exposure was classified by an occupational hygienist.

Smoking habits

The participants were asked if they had ever smoked each day during at least one month, and at what age they started and quit. They were also asked if they had smoked only cigarettes or also pipe, and how many cigarettes they had smoked per day during different age intervals. They were categorized as never-smokers or ever-smokers, and ever-smokers were divided into former or active smokers. Cumulative smoking was expressed in pack-years. The number of pack-years was first calculated for each age interval as the mean number of cigarettes smoked per day, divided by 20, and multiplied by the number of years, and then all numbers were summed.

Diet

The questionnaire included questions on diet, for example frequency of fish meals during the last year and type of fish (only from the sea or also from lakes). They were also asked if they had been a vegetarian or eaten vegetables or potatoes grown at home in the 1990s, and if they had municipal drinking water or water from their own wells.

3.1.3 Metal analyses in kidney, blood and urine

All metal analyses were performed at the Department of Occupational and Environmental Medicine at Lund University Hospital, Lund, Sweden. The concentrations of cadmium and lead in kidney cortex, and cadmium in urine and blood, were determined by inductively coupled plasma-mass spectrometry (ICP-MS; Thermo X7, Thermo Elemental, Winsford, UK) (91). Total mercury in the kidney was determined by cold vapor atomic fluorescence spectrometry (CVAFS) (92).

Cadmium, mercury and lead in the kidney

The kidney cortex biopsies were analyzed in four different rounds, and the limit of detection (LOD) was calculated as three times the SD for the blank in each round. For kidney cadmium (K-Cd), no values were below LOD (LOD 0.05, 0.03, 0.03, and 0.03 ng/sample). For kidney mercury (K-Hg) 32 values were below LOD (0.23, 0.26, 0.83, and 0.19 ng/sample), and for kidney lead (K-Pb) eleven values were below LOD (0.16, 0.15, 0.06, and 0.02 ng/sample). In order to check for accuracy an external quality control sample was analyzed six times in each round, and the results were in accordance with the target values. The dry weight kidney concentrations of metals were transformed to wet weight (ww) by multiplying by 0.18 (93). For K-Hg and K-Pb concentrations <0.01 µg/g ww, we used the value 0.01 (lowest LOD/2) in the calculations, whereas for concentrations >0.01 µg/g we used the estimate from chemical analyses. In order to assess the total amount of each metal in the kidney, we estimated the kidney weight for each donor from the body surface area and multiplied by the metal concentration (94). More details about the analyses of the kidney samples can be found in Paper I-III.

Cadmium in urine and blood

All U-Cd values in Paper III are derived from a reanalysis in 2012 of all urine samples in one batch, when all U-Cd concentrations were corrected for molybdenum oxide-based interference (95). For U-Cd below LOD (0.03 or 0.05 µg/L), we used LOD/$\sqrt{2}$ in the statistical analyses, and for B-Cd below LOD we used LOD/2 (LOD: 0.01-0.04 µg/L) depending on the data distribution (96). In order to account for variations in dilution of the urine, the U-Cd concentrations were adjusted for urinary creatinine, either by calculating the cadmium/creatinine ratio (given in µg/gC, i.e. µg cadmium/g creatinine), or by including creatinine in the multivariate model as a predictor.

3.1.4 Urine analyses

From the 109 donors with kidney metal concentrations, 106 morning urine samples and 95 24-hour urine samples were collected. Volumes were measured and sampling times were registered. Three of the 24-hour urine samples were excluded from the statistical analyses as the sample time was too short (≤15 hours), and four were excluded as they were considered false or not representative (<700 ml, N=3, and >5000 ml, N=1). The remaining 88 24-hour urine samples were included in the statistical analyses. However, as some of the biomarker analyses failed due to logistic shortcomings, the final number varies between different biomarkers.

The urine was analyzed for creatinine, calcium (Ca), albumin (Alb), alpha-1-microglobulin (A1M), beta-2-microglobulin (B2M), N-acetyl-beta-D-glucosaminidase (NAG), kidney injury molecule 1 (KIM) and retinol-binding protein (RBP). U-KIM and U-RBP analyses were performed at the Department of Occupational and Environmental Medicine, University of Gothenburg, Sweden. All other urinary biomarkers, except for cadmium, were analyzed at the Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg, Sweden. For details of the chemical analyses, see Paper II-III.

The excretion rate per hour was calculated for all biomarkers, and to account for differences in urinary dilution, they were also adjusted for urinary creatinine. For urinary calcium, correction for creatinine was a way to adjust for differences between men and women. Also, other published studies have often used urinary calcium adjusted for creatinine, as the urine samples have not been timed. For values of the renal biomarkers that were below the limit of detection, we used LOD/$\sqrt{2}$ in the statistical analyses.

3.1.5 Serum analyses

Serum samples were analyzed for ferritin, ionized calcium, parathyroid hormone, inactive and active vitamin D3 (calcidiol or 25(OH)D3, and calcitriol or 1,25$(OH)_2$D3), and cystatin C. The analyses were performed by the Department of Clinical Chemistry, Sahlgrenska University Hospital, Gothenburg, Sweden. For details of the chemical analyses, see Paper II-III.

3.1.6 Bone mineral density

In a subgroup of 67 of the 109 donors with kidney cadmium, bone mineral density (BMD; g/cm^2) of the total body, femoral neck, trochanter, lumbar spine (vertebra L2-L3) and forearm (radius) was measured by dual-energy X-ray absorptiometry (DXA) between April 2000 and December 2004. The Lunar DPX-L equipment (GE Lunar Corp.) was used.

3.1.7 Other variables

Dental amalgam surfaces

The donors were asked if they had had any dental amalgam fillings during the past year, and the total number of amalgam surfaces was counted for each participant by a physician or an occupational hygienist. In addition, the number of occlusal amalgam surfaces was counted.

Glomerular filtration rate

As part of the routine examinations before the transplantation, glomerular filtration rate (GFR) was assessed in all kidney donors. In most cases GFR was measured by Cr-EDTA clearance or iohexol clearance (mL/min/1.73 m^2 body surface area). As most other studies have used estimated GFR (eGFR), this was also calculated using the cystatin C-based CKD-EPI formula: eGFR = 127.7 * (serum cystatin C)$^{-1.17}$ * age$^{-0.13}$ * 0.91 (if female) (97).

Urinary flow rate

Urinary flow rate (mL/h) was calculated as the volume divided by the sampling time, both for overnight urine and for 24-hour urine.

Weight

Body weight was measured in kilograms after admission to the hospital before the transplantation.

Menopause

As bone resorption increases substantially in women after menopause, we wanted to correct for this in Paper II (69, 70). However, there was no information about menopause in the data set. We therefore constructed a menopause variable by assuming menopause for women >51 years old, as this is the median age of natural menopause (98).

3.2 Paper IV – the MrOS study

3.2.1 Study population and sampling

Paper IV is based on data from a cohort of 1010 elderly men in Gothenburg, Sweden. It is part of the Osteoporotic Fractures in Men (MrOS) study, which is both a cross-sectional and a prospective multicenter study with focus on bone metabolism and fractures. The participants were randomly selected from national population registries and invited to take part in the study. To be included in the study, they had to be able to walk without assistance and to give information about lifestyle factors, medical history, and medication. All study subjects gave written informed consent to participate. The study was approved by the Ethics Committee at the University of Gothenburg.

At baseline (year 2002-2004), blood and urine samples were collected, and bone mineral density was measured. The participants also underwent an examination and answered a questionnaire on smoking history, medication, physical activity etc. During the follow-up period, all new fractures were registered, first in 2009 and then in 2013. Central registers covering all

Swedish citizens were used to identify the study subjects who died during the follow-up period, and the time of death.

For the study in paper IV, cadmium could be analyzed in urine samples from 983 men. Forty-four of the subjects had very diluted urine samples (urinary creatinine <0.3 g/L) and were therefore excluded. One man was excluded due to missing urinary creatinine and one as he had not answered the questionnaire. Also, one man was excluded as he had very high urinary cadmium (9.0 μg/g creatinine), which could not be explained by smoking (he had only smoked for two years), but possibly by occupational exposure or contamination. The remaining 936 men formed the final study group in paper IV. The median age at baseline was 75 years (70.5-81). 39% were never-smokers, 53% were former smoker and 8% were current smokers.

3.2.2 Data from the questionnaire

Smoking habits

The participants were categorized either as never-smokers or ever-smokers (including former and current smokers). The variable pack-years was calculated as the mean number of cigarettes smoked per day, divided by 20, and multiplied by the number of years the person had smoked. The variable current smoking (yes = 1 or no = 0) was used instead of pack-years in some models.

Physical activity

The variable physical activity was the person's daily walking distance in kilometers/day, and was calculated as the combination of self-reported walking outdoors in daily life activities and walking as a means of exercise.

Falls

The variable falls was defined as self-reported falls during the past twelve months.

3.2.3 Cadmium and creatinine analyses in urine

At baseline, morning urine was collected from the participants and frozen. In 2012, the urine samples were analyzed for cadmium and creatinine at the Department of Occupational and Environmental Medicine at Lund University Hospital. Cadmium in urine was measured by ICP-MS (Thermo X7, Thermo Elemental, Winsford, UK). The samples were diluted 1:10 with an alkaline solution and corrected for molybdenum oxide-based interference (91). In order to assess the technical error of the measurement, the urine

samples were prepared in duplicate. The imprecision, calculated as the coefficient of variation (CV) for duplicate preparations, was 4.4%. The limit of detection (LOD) for U-Cd was 0.05 µg/L. In five of the 936 men included in the study group, U-Cd was below LOD. In these five cases, the estimate from the analysis was used (U-Cd 0.01-0.03 µg/L). Three different quality control samples were analysed. The U-Cd concentrations were adjusted for urinary creatinine and given in µg cadmium/g creatinine. Creatinine concentrations in urine were analyzed using the Jaffé method with a COBAS 6000 instrument (Roche Diagnostics, Rotkreuz, Switzerland), with a LOD of 0.1 mmol/L. More details of the analyses of cadmium in urine can be found in Paper IV.

3.2.4 Bone mineral density

Areal bone mineral density (aBMD, g/cm^2) of the total body, total hip (including femoral trochanter and femoral neck), and lumbar spine (vertebrae L1-L4) was measured at baseline by dual-energy X-ray absorptiometry (DXA). The Hologic QDR 4500/A-Delphi equipment (Hologic, Waltman, MA, USA) was used. The coefficient of variation for the measurements ranged between 0.5 and 3%. A standardized BMD (sBMD) was calculated, as the BMD measurements in other parts of the MrOS-study were made with different equipment (99-101).

3.2.5 Fractures

At the time of fracture evaluation, the radiologic archives in Gothenburg were searched for all new (incident) fractures that had occurred in the cohort after the baseline examination, using the Swedish personal identity number. The fractures were registered by date and type of fracture with a code according to the International Classification of Diseases and Related Health Problems – Tenth Revision (ICD-10). Those fractures that were reported by the study participants themselves were only included if they could be verified by physician review of radiologic reports. Vertebral fractures were only included if symptoms were reported by the participants after inclusion in the study (clinical vertebral fractures). Osteoporosis fractures were defined as fractures of the hip, distal radius, proximal humerus, pelvis, and thoracic and lumbar spine (clinical vertebral fractures). We also analyzed non-vertebral osteoporosis fractures, hip fractures and clinical vertebral fractures separately.

The following ICD-10 codes were included:

- All fractures: S02, S12, S22, S32, S42, S52, S62, S72, S82 and S92.

- Non-vertebral osteoporosis fractures: S32.1, S32.4, S32.5, S42.2, S52.5, S52.6, S72.0, S72.1 and S72.2 (fracture of sacrum, acetabulum, pubis, upper end of humerus, lower end of radius, lower end of both ulna and radius, neck of femur, pertrochanteric fracture and subtrochanteric fracture).

- Hip fractures: S72.0, S72.1 and S72.2 (fracture of neck of femur, pertrochanteric fracture and subtrochanteric fracture).

- Clinical vertebral fractures: S22.0, S22.1 and S32.0 (fracture of thoracic vertebra, multiple fractures of thoracic spine, and fracture of lumbar vertebra).

- Other fractures: All fractures except clinical vertebral fractures and non-vertebral osteoporosis fractures.

As some participants had more than one type of fracture during the follow-up period, on the same day or on different days, the same person could be included in more than one fracture subgroups. The risk time (in days) for the first incident fracture in each fracture group was calculated from the date of the baseline examination until the date of the fracture, the date of death or the end of the follow-up time. As a result the same person could have different risk times in different fracture groups.

3.2.6 Other variables

Body mass index

Body mass index (BMI) was calculated as the person's weight in kilograms divided by height in square meters (kg/m^2). Weight and height were measured twice at baseline, and BMI was calculated from the means.

Estimated GFR (eGFR)

GFR was estimated using a cystatin C-based formula: eGFR = 79.901 * (serum cystatin C)$^{-1.4389}$ (102). Serum cystatin C was measured using the

Hitachi Modular P analyzer (reagents and calibrators from Daco A/S, Copenhagen), with a total imprecision of 2.1%.

Serum/plasma analyses

Blood samples were collected between 8:00 and 8:30 a.m. after at least 10 hours of fasting and non-smoking, and immediately frozen. Serum sex hormone-binding globulin (SHBG) was measured using an immuno-radiometric assay (Orion Diagnostica, Espoo, Finland; LOD 1.3 nM; intra-assay CV 3%; interassay CV 7%). A validated gas chromatography-mass spectrometry (GC-MS) system was used for the analyses of testosterone (LOD 0.05 ng/ml; intra-assay CV 2.9%, interassay CV 3.4%), and estradiol (LOD 2.0 pg/ml, intra-assay CV 1.8%, interassay CV 1.7%). Plasma levels of osteocalcin were measured using monoclonal antibodies against human osteocalcin and detected by electrochemiluminence (Elecsys N-MID Osteocalcin Cal-Set, Roche Diagnostics, Indianapolis, IN, USA). Serum levels of the N-terminal propeptide of type I procollagen (PINP), a bone formation marker, were measured by a radioimmunoassay with polyclonal antibodies against human procollagen I (PINP RIA; Orion Diagnostica, Espoo, Finland).

3.3 Statistical analyses

All statistical analyses were performed using the SAS software package (versions 9.2 and 9.4). P-values <0.05 were considered statistically significant in a two-tailed test in all papers.

Paper I

Spearman's correlation analysis was performed in order to evaluate associations between single variables (correlation coefficient r_s). Differences between groups were assessed by Wilcoxon rank sum test, as the concentrations of cadmium, mercury and lead were somewhat skewed. Multiple linear regression was used to evaluate predictors of the levels of heavy metals in the kidney. Both untransformed and log-transformed concentrations of heavy metals were used.

Paper II

Spearman's and Pearson's correlation analyses were performed in order to evaluate associations between single variables (correlation coefficients r_s and r_p, respectively). Student's t-test was used to calculate differences between independent groups. For related samples, a paired t-test was performed.

Kidney cadmium was treated both as a continuous and as a categorical variable. Categorical K-Cd was defined as "high" in those above the median K-Cd (12.9 μg/g ww) and "low" in those below the median. Multiple linear regression analyses were performed to examine associations between continuous urinary calcium (response variable) and predictor variables. A regression model was constructed after backward elimination of non-significant variables:

$y = \alpha + \beta1 * x + \beta2 * age + \beta3 * sex + \beta4 * body\ weight + \beta5 * menopause + \beta6 * ionized\ S\text{-}Ca + \beta7 * urinary\ flow\ rate + \beta8 * vitamin\ D\ (25(OH)D3) + \varepsilon$

y = U-Ca; x = K-Cd; α = intercept; β = regression coefficient; ε = error

Logistic regression was used to assess associations between dichotomized urinary calcium (high/low U-Ca, where "high U-Ca" was defined as the fourth quartile of U-Ca) and predictors. K-Cd and body weight were the only predictor variables left in the model after stepwise selection (with p=0.1 as the upper limit for inclusion in the model). Multiple linear regression was also used to calculate associations between BMD and predictor variables:

$y = \alpha + \beta1 * x + \beta2 * age + \beta3 * sex + \beta4 * body\ weight + \beta5 * menopause + \beta6 * smoking + \beta7 * vitamin\ D\ (25(OH)D3) + \varepsilon$

y = BMD; x = K-Cd; α = intercept; β = regression coefficient; ε = error

Paper III

Spearman's correlation analysis was performed in order to assess associations between single variables. Differences between groups were assessed by Student's t-test for independent groups, and a paired t-test for related samples. As in paper II, kidney cadmium was treated both as a continuous and as a categorical variable, where "high K-Cd" was above the median (>12.9 μg/g ww) and "low K-Cd" was below the median. Multiple linear regression was used to calculate associations between biomarkers of kidney function (response variables) and predictor variables. As the distributions of several renal biomarkers were skewed, the natural logarithm of the biomarkers (Ln y) was used in the multiple linear regression models:

Equation 1: $Ln\ y = \alpha + \beta1 * x + \beta2 * age + \beta3 * sex + \beta4 * body\ weight + \beta5 * smoking + \beta6 * pack\text{-}years\ (+ \beta7 * urinary\ flow\ rate) + \varepsilon$

y = biomarker of kidney function; x = K-Cd, B-Cd or U-Cd; α = intercept; β = regression coefficient; ε = error.

For K-Cd and B-Cd, equation 1 was used. When the dependent variable was measured in urine, urinary flow rate was included in the model.

For U-Cd, three different models were used: model 1: as equation 1 but without urinary flow rate, model 2: as equation 1 but also including urinary creatinine, model 3: as equation 1 including urinary flow rate.

Paper IV

Associations between single variables were calculated using Spearman's correlation analysis. Differences between groups were assessed using Student's t-test. Associations between U-Cd and BMD were calculated using multiple linear regression for continuous U-Cd, and general linear models for quartiles of U-Cd, in a model adjusted for age, BMI, smoking (pack-years) and physical activity (daily walking distance):

$$y = \alpha + \beta 1 * x + \beta 2 * \text{age} + \beta 3 * \text{BMI} + \beta 4 * \text{pack-years} + \beta 5 * \text{physical activity} + \varepsilon$$

y = BMD; x = U-Cd; α = intercept; β = regression coefficient; ε = error

Associations between U-Cd and incident fractures were analyzed by Cox proportional hazards regression, in four different models. Hazard ratios (HR) were calculated for incident fractures by quartiles of U-Cd, with the lowest quartile as the reference, or per 1 µg Cd/g creatinine. Model 1 only included U-Cd. Model 2a also included age, pack-years, BMI and physical activity. Model 2b was as model 2a but included current smoking instead of pack-years. Model 3 was as model 2a but also included sBMD of the femoral neck. In addition, models including eGFR, falls, or SHBG were tested, but these covariates were not included in the final models.

4 RESULTS

4.1 Paper I

4.1.1 Cadmium, mercury and lead in kidney cortex

Levels of cadmium, mercury and lead in kidney cortex biopsies are shown in Table 1 below (based on Table 2, Paper 1). Women had significantly higher cadmium concentrations in kidney cortex (K-Cd) than men (p=0.01), and also higher total amount of cadmium in the kidney (calculated from the estimated kidney weight). Kidney mercury levels (K-Hg) were slightly higher in women than in men, but the difference was not statistically significant. There were no significant differences in kidney lead concentrations (K-Pb) between men and women.

Table 1. Kidney concentrations (µg/g ww) of cadmium, mercury, and lead. Based on Table 2, Paper 1.

	All		**Women**		**Men**	
	N	*Median (range)*	*N*	*Median (range)*	*N*	*Median (range)*
K-Cd, all	109	12.9 (1.5-55.4)	60	14.7 (1.5-55.4)	49	10.9 (1.6-31.7)
K-Cd, ever-smokers	68	16.7 (1.5-55.4)	38	18.6 (1.5-55.4)	30	15.6 (3.1-31.7)
K-Cd, never-smokers	41	8.3 (1.6-30.3)	22	10.5 (3.0-27.9)	19	5.6 (1.6-30.3)
K-Hg	109	0.21 (<LOD-2.4)	60	0.25 (<LOD-2.4)	49	0.18 (<LOD-1.2)
K-Pb	109	0.08 (<LOD-2.2)	60	0.07 (<LOD-2.2)	49	0.08 (<LOD-1.2)

4.1.2 Impact of exposure sources and background factors

Occupational exposure

Only one of the 109 subjects with kidney metal concentrations had been occupationally exposed to cadmium (K-Cd 4.5 µg/g ww). He had worked in a smelter and also been exposed to mercury and lead. Four other subjects had probable or low occupational exposure to mercury, and all of these also had amalgam fillings. For lead, four other subjects had been occupationally exposed, and another six had probable or low occupational exposure.

Cadmium

Ever-smokers had significantly higher levels of K-Cd than never-smokers (median K-Cd 16.7 and 8.3 µg/g ww, respectively), both in women and men (Table 1 and Figure 3). Never-smoking women had higher K-Cd than never-smoking men (p=0.03). In univariate analyses (Spearman correlation), K-Cd was positively associated with the number of pack-years (r_s=0.51, p<0.0001). K-Cd was also positively associated to age (r_s=0.28, p=0.003), both in ever- and never-smokers. However, after age about 65 years (N=5), K-Cd instead seemed to decline (Figure 3).

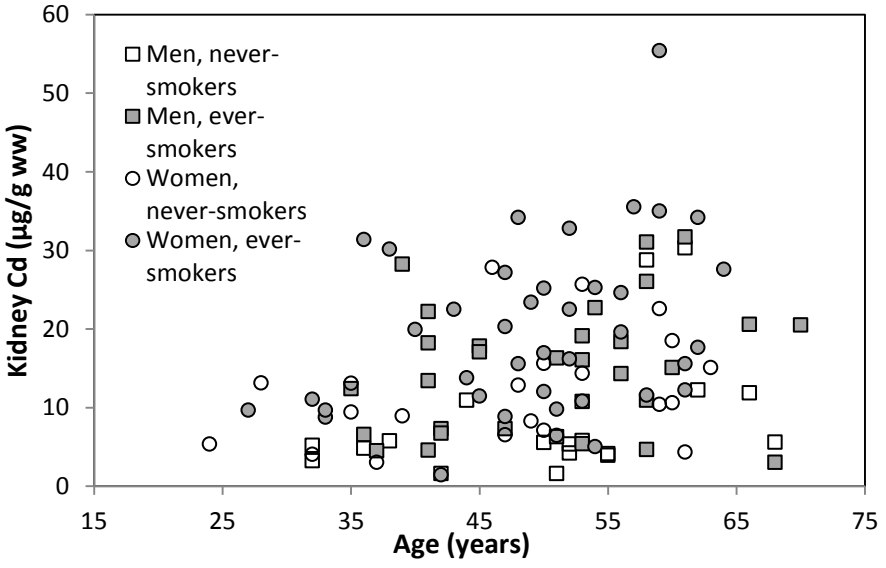

Figure 3. Kidney cadmium concentrations as a function of age, sex, and smoking. Based on Figure 1, Paper 1.

K-Cd was negatively associated with body weight (r_s= –0.29, p=0.002). Serum-ferritin, a marker of the total amount of iron stored in the body, was negatively associated to K-Cd in our study, but the association was not statistically significant (r_s= –0.19, p=0.05).

In multivariate analyses, we excluded the five participants aged >65 years as K-Cd is expected to decrease at old age (7). The impact of the independent variables age, sex, pack-years, low iron stores in women (S-ferritin category, S-ferritin ≤30 µg/L), and body weight on the dependent variable K-Cd was tested in multiple linear regression analyses with stepwise selection. For untransformed K-Cd (µg/g ww), a significant effect of age, sex and pack-years was found. For log-transformed K-Cd, we found a significant effect of age, pack-years, iron stores and body weight, but not sex. For estimated total kidney cadmium, and also log-transformed total kidney cadmium, a significant effect of age, pack-years and iron stores, but not sex or weight, was found. Both K-Cd and total kidney cadmium was higher in never-smoking women than in never-smoking men after including only those with normal iron stores, but the difference was not statistically significant. Two different models for K-Cd were constructed based on the results described above. All covariates were significant in those two models:

Model 1: K-Cd = – 6.1 + 0.31 * age + 0.41 * pack-years + 5.0 * sex

Model 2: K-Cd = 2.0 + 0.39 * age + 0.37 * pack-years – 0.13 * body weight + 4.5 if S-ferritin ≤30

Mercury

In univariate analyses (Spearman correlation), K-Hg was positively correlated with the total number of amalgam surfaces (r_s=0.62, p<0.0001), as well as the number of occlusal amalgam surfaces (r_s=0.54, p<0.0001). In the multiple regression analyses, log K-Hg, as well as log total kidney Hg, was significantly associated with the number of amalgam surfaces (p<0.0001), but not with age, sex, body weight, or fish consumption, after stepwise selection.

Lead

No significant associations were found between K-Pb and the independent variables (age, pack-years, years of smoking, and body weight), neither univariate nor multivariate. The results were similar with log-transformed K-Pb or total kidney Pb as the dependent variable, and also did not change significantly after excluding participants with occupational exposure.

4.2 Paper II

4.2.1 Excretion of calcium in urine

The excretion of calcium (U-Ca) was significantly higher in 24-hour urine than in overnight urine, both per hour and normalized for creatinine ($p<0.001$). The excretion rate of U-Ca (mmol/h) was positively correlated with the excretion rate of creatinine in urine (mmol/h), both in 24-hour and overnight urine ($r_p=0.45$ and $r_p=0.35$, respectively, $p<0.001$). In the overnight sample, diuresis (urinary flow rate, mL/h) was positively correlated both with U-Ca and creatinine excretion rates ($r_p=0.30$ and $r_p=0.28$, respectively, $p<0.01$). Men excreted significantly more U-Ca per hour than women in both samples. On the other hand, there was a trend for women to have higher excretion of U-Ca adjusted for creatinine (non-significant). There was no significant difference in urinary flow rate between men and women.

In univariate analyses, U-Ca excretion (per hour and adjusted for creatinine) was positively correlated with ionized S-Ca. U-Ca adjusted for creatinine was also positively correlated with age and menopause, but negatively correlated with weight. U-Ca excretion per hour was instead positively correlated with weight.

4.2.2 Associations between kidney cadmium and calcium in urine

In univariate analyses, significant positive correlations were found between continuous K-Cd and U-Ca normalized for creatinine, both in 24-hour and overnight urine. When the analyses were repeated for women and men separately, there were significant associations between K-Cd and U-Ca in women, but not in men.

When we used categorical K-Cd ("high K-Cd" above the median 12.9 µg/g ww, or "low K-Cd" ≤12.9 µg/g ww), we found that donors with high K-Cd excreted significantly more U-Ca normalized for creatinine, both in 24-hour urine (Figure 4) and overnight urine. This was mainly due to a significant difference in U-Ca excretion in women, both creatinine-adjusted and per hour. Again, no significant difference was seen in men.

Both ever-smokers and never-smokers with high K-Cd had significantly higher excretion of U-Ca normalized for creatinine in 24-hour urine, but not in overnight urine.

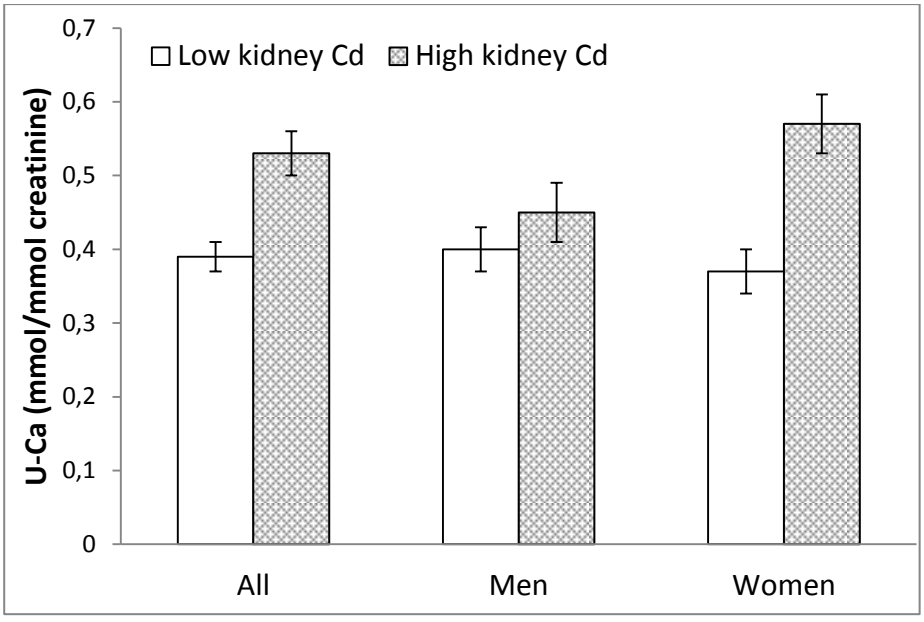

Figure 4. Mean urinary calcium excretion in 24-hour urine (mmol/mmol creatinine) at kidney cadmium levels below or above the median (low/high kidney Cd). Based on Figure S2, Paper 2.

In multivariate analyses, with continuous U-Ca as the dependent variable, a regression model including categorical K-Cd, age, sex, body weight, menopause, ionized S-Ca, urinary flow rate and vitamin D (25(OH)D3) was constructed after backward elimination. We found that U-Ca excretion in the 24-hour sample was significantly associated with high K-Cd (both the excretion normalized for creatinine and per hour). The same association was found in women, but not in men. In the 24-hour sample, high K-Cd also increased the odds of having high U-Ca (in the fourth quartile) normalized for creatinine (OR 5.5, 95% CI 1.6-19). No significant associations were found in the overnight sample. Likewise, there was no significant effect of K-Cd as a continuous variable in the multivariate analyses.

4.2.3 Associations between kidney cadmium and BMD

In univariate analyses, significant negative correlations were found between high K-Cd and BMD for total body, lumbar spine, femoral trochanter, and forearm (distal radius). However, no significant associations were found between K-Cd and BMD in the multivariate analyses, in a regression model including categorical K-Cd, age, sex, body weight, menopause, smoking and

vitamin D (25(OH)D3). In univariate analyses, BMD for most sites was negatively correlated with U-Ca normalized for creatinine, but in the multiple regression analyses these associations were no longer significant.

4.3 Paper III

4.3.1 Associations between cadmium and renal biomarkers

Univariate analyses

There were significant positive correlations between K-Cd and U-A1M normalized for creatinine (U-A1MCrea) both in 24-hour and overnight (ON) urine (Figure 5 and 6). We also found significant positive correlations between K-Cd and U-NAG as well as U-KIM normalized for creatinine (U-NAGCrea and U-KIMCrea) in 24-hour urine. In addition, K-Cd was positively correlated with ON U-RBP normalized for creatinine (U-RBPCrea). No significant correlations were found between K-Cd and GFR, S-cystatin C, U-Alb, U-B2M, ON U-NAG, ON U-KIM or 24-hour RBP.

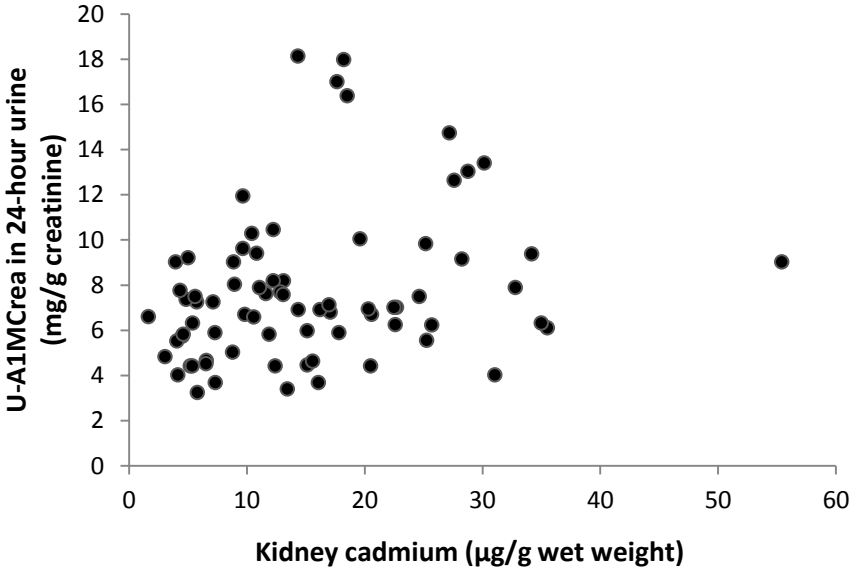

Figure 5. Excretion of alpha-1-microglobulin (mg/g creatinine) in 24-hour urine as a function of kidney cadmium.

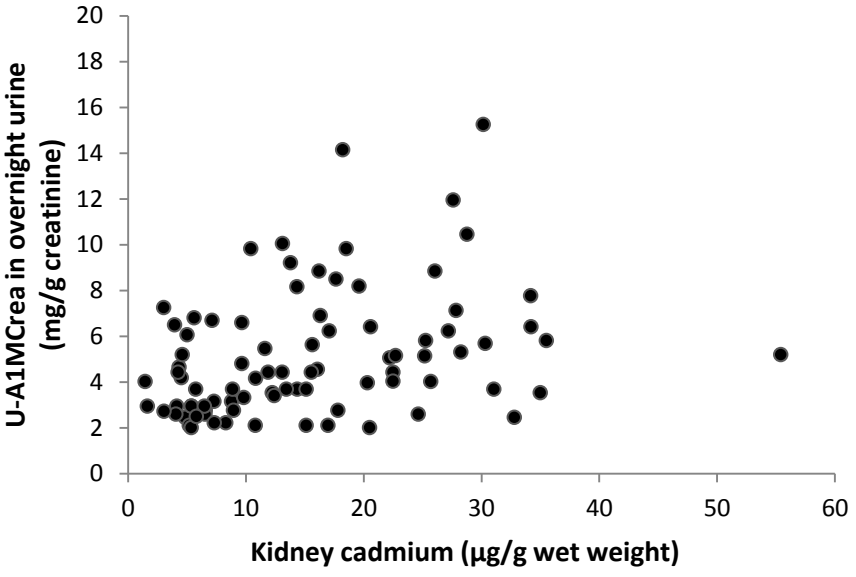

Figure 6. Excretion of alpha-1-microglobulin (mg/g creatinine) in overnight urine as a function of kidney cadmium.

Significant negative correlations were found between U-Cd and eGFR, but not with mGFR, and the correlation between eGFR and mGFR was poor (r_s=0.16, p=0.12).

Donors with high K-Cd excreted significantly more 24-hour U-A1MCrea, ON U-A1MCrea and ON U-A1M/h than those with low K-Cd (≤ the median 12.9 µg/g ww).

Multivariate analyses including K-Cd

Significant positive associations were found between lnU-A1M in ON urine and continuous K-Cd in a multiple regression model including age, sex, weight, smoking (never/ever), pack-years and urinary flow rate (Table 2). There was also a significant association between ON lnU-A1M (both normalized for creatinine and per hour) and categorical K-Cd.

We also did separate analyses for men and women, using the same regression model. In men, significant positive associations were found between lnU-A1M and K-Cd, both in ON and 24-hour urine (normalized for

creatinine and per hour). No significant associations between lnU-A1M and K-Cd were found in women.

As for the other renal biomarkers, and GFR, there were no significant associations with K-Cd.

Multivariate analyses including B-Cd

ON lnU-A1M (per hour) was significantly and positively associated with B-Cd, using the same linear regression model but with B-Cd instead of K-Cd (Table 2). There were no other significant associations between B-Cd and renal biomarkers, except for ON lnU-RBP.

Table 2. Associations between K-Cd or B-Cd and ln-transformed markers of kidney function in a multiple regression model including age, sex, weight, smoking (never/ever), and pack-years. For renal biomarkers measured in urine, urinary flow rate was also included in the model. Based on Table 2, Paper 3.

	K-Cd (µg/g ww)	**B-Cd (µg/L)**
Dependent variable	*β, SE (p value)*	*β, SE (p value)*
GFR (mL/min/1.73 m^2)	-0.001, 0.002 (0.52)	-0.006, 0.03 (0.86)
S-cystatin C (mg/L)	-0.003, 0.003 (0.32)	-0.08, 0.06 (0.19)
24h U-Alb (mg/h)	-0.007, 0.007 (0.34)	-0.06, 0.013 (0.65)
ON U-Alb (mg/h)	-0.007, 0.006 (0.31)	0.18, 0.13 (0.16)
24h U-A1M (mg/h)	0.007, 0.004 (0.09)	0.13, 0.07 (0.09)
ON U-A1M (mg/h)	0.009, 0.004 (**0.04**)	0.20, 0.08 (**0.02**)
24h U-KIM (ng/h)	-0.002, 0.008 (0.78)	-0.09, 0.14 (0.56)
ON U-KIM (ng/h)	-0.012, 0.008 (0.15)	0.014, 0.17 (0.40)
24h U-RBP (ng/h)	-0.002, 0.008 (0.85)	-0.034, 0.15 (0.82)
ON U-RBP (ng/h)	-0.006, 0.008 (0.47)	0.32, 0.16 (**0.04**)

Multivariate analyses including U-Cd

Significant positive associations were found between lnU-A1M and U-Cd, both in ON and 24-hour urine (Table 3). In a model including urinary flow rate (model 3), the association was significant both with U-Cd excretion per hour and U-Cd concentration (µg/L). The association was seen for lnU-A1M/h as well as lnU-A1MCrea. Some associations were found between U-Cd and other biomarkers (lnU-Alb and lnU-RBP), but only in ON urine. No significant associations were found with GFR. A1M can also be increased in glomerular disease, but we found no association with GFR.

Effect of smoking

There was no correlation between pack-years of smoking and A1M-excretion. In multivariate analyses with A1M excretion per hour as the response variable, beta coefficients for categorical smoking and pack-years were negative and not significant. The multivariate analyses were repeated in never-smokers only, and again we found that A1M excretion rates in both samples were significantly associated with K-Cd. We also found that the beta coefficients for K-Cd were larger in never-smokers than in the total study group. In the multivariate models with B-Cd or U-Cd, the beta coefficients for these predictors were also larger in never-smokers, but they were not statistically significant.

4.4 Paper IV

4.4.1 Associations between urinary cadmium and BMD

In the cohort of elderly men in Gothenburg, U-Cd (as a continuous variable) was significantly and negatively correlated with total body BMD, and sBMD for total hip, trochanter, femoral neck, and lumbar spine. In addition, total body BMD as well as sBMD for all sites was significantly lower in the highest quartile of U-Cd compared to the lowest.

In multivariate analyses, BMD was still significantly lower in the highest quartile of U-Cd, compared to the lowest, in a model including age, BMI, pack-years and physical activity. The associations between BMD and continuous U-Cd were not significant in the same multivariate model.

Table 3. Associations between U-Cd and selected ln-transformed markers of kidney function in a multiple regression model including age, sex, weight, smoking (never/ever), and pack-years. Model 2 also includes creatinine, and model 3 also includes urinary flow rate. P-value only given if p<0.1. Based on Table 2, Paper 3.

Dependent variable		**24h U-Cd (µg/24h)** *β, SE (p value)*	**24h U-Cd (µg/L)** *β, SE (p value)*	**ON U-Cd (µg/h)** *β, SE (p value)*	**ON U- Cd (µg/L)** *β, SE (p value)*
24h U-Alb (mg/h)	1	0.24, 0.33	-0.11, 0.84		
	2	0.24, 0.35	-0.18, 0.52		
	3	0.27, 0.34	0.02, 0.51		
ON U-Alb (mg/h)	1			13, 5.6 (0.054)	0.06, 0.19
	2			13, 6.7 (0.06)	0.07, 0.23
	3			13, 6.7 (0.06)	0.16, 0.22
24h U-A1M (mg/h)	1	0.45, 0.24 (0.06)	-0.32, 0.32		
	2	0.70, 0.22 (**0.002**)	0.28, 0.35		
	3	0.64, 0.18 (<**0.001**)	0.59, 0.29 (**0.045**)		
ON U-A1M (mg/h)	1			22, 6.6, (**0.002**)	-0.51, 0.18, (**0.008**)
	2			21, 4.5, (<**0.001**)	0.33, 0.17, (0.06)
	3			17, 4.0, (<**0.001**)	0.32, 0.15, (**0.03**)
24h U-KIM (ng/h)	1	0.42, 0.36	0.86, 0.49 (0.08)		
	2	0.23, 0.36	0.33, 0.55		
	3	0.38, 0.36	0.74, 0.55		
ON U-KIM (ng/h)	1			14, 8.6	0.01, 24
	2			14, 8.7	-0.01, 0.30
	3			13, 8.6	0.26, 0.28
24h U-RBP (ng/h)	1	0.005, 0.37	-0.40, 0.50		
	2	-0.03, 0.38	-0.65, 0.57		
	3	0.04, 0.37	-0.27, 0.56		
ON U-RBP (ng/h)	1			17, 8.5 (0.05)	-0.28, 0.24
	2			13, 8.4	-0.20, 0.24
	3			15, 8.2 (0.08)	0.11, 0.27

However, if the smoking variable (pack-years) was excluded from the model, the associations between continuous U-Cd and total body BMD, total hip sBMD, and trochanter sBMD were significant. When the variable pack-years, but not U-Cd, was included in the model, there were significant associations with BMD for the same sites.

4.4.2 Associations between urinary cadmium and fractures

The risk (HR) of getting a first, incident fracture was calculated from baseline (2002-2004) until the end of 2013, using Cox proportional hazards regression. In the crude (unadjusted) model, the HRs of all incident fractures, all osteoporosis fractures, non-vertebral osteoporosis fractures and hip fractures were significantly increased in the fourth quartile of U-Cd, when the first quartile was used as the reference. However, in the multivariate model, adjusted for age, pack-years, BMI, and physical activity (model 2a), only the HR for non-vertebral osteoporosis fractures remained significant in the fourth quartile (both 2009 and 2013).

When the variable current smoking (yes/no) was used instead of pack-years in the multivariate model (model 2b), the HR in the fourth quartile of U-Cd was significant also for all fractures and all osteoporosis fractures. U-Cd was not associated with sex hormones, SHBG, osteocalcin, procollagen, or eGFR in the univariate analyses (Spearman correlation). There was however a positive significant correlation between U-Cd and "falls during the last year". When the analyses were repeated including also the covariate "falls during the last year", eGFR, or SHBG in model 2, the results (HRs) were very similar both at first and second follow-up, and these covariates were not included in the final model.

When we added sBMD of the femoral neck to the multivariate model (model 3), the HR for non-vertebral osteoporosis fractures was still significant in the fourth quartile of U-Cd at first follow-up (2009), but not at second follow-up (2013). Significant HRs were found in quartile 3 for all osteoporosis fractures and vertebral fractures in 2013, but HRs in quartile 4 were non-significant for all fracture groups.

For more details, see Paper IV.

5 DISCUSSION

5.1 Discussion of the results in Paper I-IV

5.1.1 Kidney metal levels (Paper I)

Kidney cadmium – Effects of smoking

The kidney cadmium levels in the TINA study (Paper I-III) were similar to those reported in a Swedish autopsy study from 1998 by Friis et al., but lower than the levels in a Swedish study from 1976 by Elinder et al., especially in those younger than 50 years (7, 9). Higher smoking rates in Sweden in the 1970s can probably explain most of this difference. In the multivariate regression model, K-Cd levels increased with 4 µg/g per 10 pack-years of smoking in our study, compared to 6 µg/g in the study from 1976 (7). The reason for this difference is not clear, but it might be the result of lower levels of cadmium in modern cigarettes, changes in smoking technique, or differences in the reporting of smoking habits.

For never-smokers, who are exposed mainly via their diet, the K-Cd levels in our study were only marginally lower compared to the 1970s, and it is not clear whether cadmium intake in Sweden has decreased. In a study from 1979 by Kjellstrom, the average daily intake of cadmium via diet was found to be about 17 µg/day (103). Currently, the average total daily intake is between 10 and 20 µg/day in Sweden, and the levels of cadmium in food do not seem to decline (22, 33, 77, 104, 105).

Kidney cadmium – differences between men and women

Women had higher K-Cd levels than men in our study, which is consistent with previous Swedish studies (7, 9, 34). This difference between men and women is thought to be the result of increased gastrointestinal absorption in those with iron deficiency, i.e. predominantly women (34-36). In our study, the difference was most prominent in never-smokers, which supports this theory. We also measured serum ferritin (S-ferritin) in order to estimate the body iron stores, and categorized those with S-ferritin ≤30 µg/L as persons with low iron stores (106). A significant effect of low S-ferritin was found on log-transformed K-Cd, as well as on total kidney cadmium (both untransformed and log-transformed), in multivariate regression with stepwise selection including age, sex, pack-years, S-ferritin category and body weight. For untransformed K-Cd, only age, sex and pack-years remained significant after stepwise selection. However, in a final model

where sex was replaced by body weight and S-ferritin category, significant effects were found for all independent variables, with an increase in K-Cd of 4.5 µg/g ww in those with low iron stores. There was a negative effect of body weight, with a decrease in K-Cd of 0.13 µg/g ww per kilo body weight, probably because of the positive association usually found between body surface area and kidney weight (94). Men also had higher body weight, and estimated kidney weight, than women in our study. When estimated total kidney cadmium was used as the dependent variable, there was no significant effect of sex or body weight after stepwise selection. Thus, low kidney weight and low iron stores can probably explain most of the differences in kidney cadmium concentrations between men and women in this study.

Kidney mercury

Median K-Hg was 0.21 µg/g (ww) and mean K-Hg was 0.32 µg/g in our study. This is similar to the results of a small Swedish autopsy study by Nylander and Weiner 1991, where median K-Hg was 0.89 µmol/kg (~0.18 µg/g, ww) and mean 1.4 µmol/kg (~0.28 µg/g) in subjects from the general population (107). In a German autopsy study from 1992, arithmetic mean K-Hg was 0.06 µg/g (GM 0.04 µg/g, ww) in those with ≤2 teeth with amalgam fillings, and 0.51 µg/g (GM 0.37 µg/g) in those with >10 teeth with amalgam fillings (6). As has previously been reported for mercury in urine, the between person variability in K-Hg was substantial in our study with a GSD of 4.0 (108-110).

K-Hg was mainly associated with the number of amalgam fillings, but not with fish consumption. The association between amalgam fillings and K-Hg is consistent with previous knowledge, as it is well-known that elemental mercury can be released from amalgam fillings and absorbed mainly via inhalation (3, 27). MeHg in ingested fish is almost completely absorbed in the gut, and after demethylation some of it is accumulated in the kidney and other organs. The absence of an association between fish intake and K-Hg in our study could be due to a much larger influence of Hg from dental amalgam fillings, and/or shortcomings in the accuracy of the data on fish intake. Only 18% of the donors with a kidney biopsy reported to have more than one fish meal per week, and 13% had less than one fish meal per month. In populations with higher intake of MeHg, the impact on K-Hg levels might be more evident. In a study by Johansen et al. (2007) from Greenland, where the traditional diet of fish, seafood and sea animals contains high levels of MeHg, mean K-Hg was 1.4 µg/g ww (14). As with cadmium, women in our study had somewhat higher K-Hg than men, probably due to smaller kidneys, as there was no effect of sex on log-transformed total kidney Hg.

Kidney lead

The median K-Pb level in our study was 0.08 µg/g (mean 0.18 µg/g, ww), which is lower than the K-Pb levels in autopsy studies from the 1980s and 1990s (4, 10, 11). The reason for the decline in K-Pb is probably due to the gradual reduction of lead in petrol in Sweden, and from 1995 completely lead-free petrol, as this was previously the main source of exposure to lead (111). However, some of the decrease might be the result of reduced exposure from other sources, such as food cans. The results in our study are in agreement with a previous study by Strömberg and colleagues (2008), who reported a decline in B-Pb levels in Swedish children since 1978, and a study by Wennberg et al. (2006), who found decreasing levels of Ery-Pb from 1990 to 1999 in northern Sweden (105, 111). However, as the K-Pb levels in our study were very low and the biopsy samples were small, the analytical precision is limited, and the results must be interpreted with caution.

5.1.2 Cadmium, kidney and bone (Papers II-IV)

It is well-known since a long time that exposure to high levels of cadmium can cause negative effects on both bone and kidney function, as previously described in the introduction (subchapter 1.2-1.4). Bone effects, such as an increased risk of low BMD, osteoporosis and fragility fractures, and an increased excretion of proteins in urine, have later been confirmed also at lower levels of exposure to cadmium (59, 76, 77, 79-84, 89, 112-115). However, it is still uncertain at what levels cadmium can affect kidney and bone. One aim of this thesis was to study the effects of cadmium at the low levels found in the general population in Sweden. Furthermore, the mechanism behind the effect of cadmium on bone is still unclear. Associations between cadmium and calcium excretion are interesting as calcium is an important component in bone, but can also be used as a marker of renal tubular function. In previous studies by Wu et al. (2001) and Buchet et al. (1990), the authors found positive associations between U-Cd and U-Ca excretion (90, 116). In Paper II-IV, we studied the associations between cadmium exposure and urinary calcium (Paper II), multiple renal biomarkers (Paper III), BMD (Paper II and IV), and incident fractures (Paper IV). Kidney cadmium was used as a biomarker of the body burden of cadmium in Paper II and III, eliminating the risk of associations caused by influence of diuresis or co-excretion for other reasons (60-62). In Paper IV we only had access to cadmium in urine, but as we studied associations with BMD and fractures, co-excretion was not a problem.

5.1.3 Kidney cadmium and calcium in urine (Paper II)

Male donors in our study excreted significantly more U-Ca per hour than the female donors in both samples, which is consistent with previous knowledge (117, 118). After adjustment for creatinine, there was however a trend for women to have higher U-Ca excretion than men. This was probably due to lower U-creatinine concentrations in women, as a result of lower muscle mass.

Significant positive associations were found between urinary calcium excretion and kidney cortex cadmium concentrations in our study, both in correlation analyses and in multivariate regression models. The associations were mainly due to significant differences in women, while no significant differences were found in men. This difference between men and women has previously been reported by Nambunmee et al. (2010) in a study of residents in Thailand with elevated U-Cd levels (119). In that study, the fractional U-Ca excretion (as well as the levels of renal and bone markers) increased with rising U-Cd especially in women. As in our study, women in the Thai study had higher creatinine-adjusted U-Ca excretion than men. In addition, the fractional U-Ca excretion was positively correlated with the excretion of bone resorption markers in the Thai study, and the levels of bone resorption markers were higher in women. These results suggest that women could be at higher risk for Cd-induced effects on bone metabolism. The reason for this could be for example differences in hormonal status and/or iron deficiency (35, 36, 120). In a study of patients with Itai-itai disease in Japan 1967-1993, the authors found that 98% of the patients with Itai-itai, and 85% with suspected disease, were women (121). Experimental animal studies also support that the female skeleton is more susceptible than the male to cadmium exposure, especially during periods of increased bone turnover, such as during pregnancy, lactation, and after menopause (85).

In our study, donors with K-Cd above the median (high K-Cd) had significantly higher excretion of U-Ca normalized for creatinine than those with low K-Cd, both in 24-hour and ON urine. In women, there was also a significantly higher U-Ca excretion per hour in those with high K-Cd, in both samples. In the multivariate model, a significant effect of categorical K-Cd on U-Ca was only seen in 24-hour urine (per hour and normalized for creatinine) but not in ON urine. The reason for this is probably that the 24-hour sample gives a more reliable value of the mean excretion of Ca in urine, as it has been collected during a longer period of time.

When U-Cd was used as a continuous variable, no significant effects on U-Ca were seen in the multivariate models. This could be due to a threshold effect, i.e. that very low levels of K-Cd have no effect on the U-Ca excretion. The presence of a threshold might explain why an effect was found when K-Cd was dichotomized (high/low).

In addition to K-Cd, the covariates age, sex, weight, menopause, ionized S-Ca, urinary flow rate, and vitamin D (25(OH)D3) were included in the final multivariate model, which was constructed after backward elimination. The variables smoking, serum PTH and $1,25(OH)_2D3$ were excluded as they were not significantly associated with U-Ca excretion in the analysis. We had no information on calcium intake from the diet, which may also affect the calcium levels.

Many different mechanisms behind the effect of cadmium on bone, and the association with U-Ca excretion, have been suggested. One possible mechanism is that cadmium causes early damage to the renal tubules, resulting in decreased reabsorption of Ca and thus increased U-Ca excretion, or a direct effect of cadmium on bone, possibly by affecting bone resorption (34, 59, 79, 86, 88, 89, 116, 122). Results from experimental studies performed in bone organ and cell culture systems support the direct act of cadmium on bone, affecting both bone resorption and bone formation (85). Increased U-Ca excretion can thus either be the result of or the cause of an increased bone resorption, which may lead to lower BMD, osteoporosis and an increased risk of fragility fractures. There is also a possibility of reverse causation, where the loss of Ca in urine results in a tendency towards lower S-Ca, which leads to an upregulation of metal transporters in the intestine, causing increased absorption of both Ca and Cd (123, 124).

5.1.4 Kidney cadmium and BMD (Paper II)

Significant negative associations were found between K-Cd and BMD in the univariate analyses, which is in accordance with the hypothesis that cadmium increases the risk for osteoporosis. However, no significant associations were found in the multiple regression analyses. There are several possible reasons for this lack of an association after adjustment for age, sex, smoking and vitamin D. One possible explanation is that we had limited power to find an association due to the small study group with BMD measurements (67/109 donors). The donors were also relatively young, with a median age of 51, and the effect of cadmium on BMD might be more pronounced at higher ages. In addition, we had no information about some other covariates such as menopausal age, previous fractures, osteoporosis in

the family history, or physical activity. As for menopausal age, we assumed menopause for women aged >51 years, as this is the median age for menopause, but this is a rough estimate.

5.1.5 Cadmium and kidney function (Paper III)

In summary, in our study we found positive associations between cadmium in kidney, blood and urine and the excretion of the LMW protein alpha-1-microglobulin (A1M) in urine, after adjustment for possible confounders and effect modifiers. We believe that this might be a causal association, i.e. that the increased U-A1M excretion is a result of cadmium toxicity, possibly due to decreased reabsorption of A1M in the proximal renal tubules. As we measured cadmium not only in urine and blood, but also in kidney cortex, we could get around the problem with co-excretion of cadmium and proteins in urine due to physiological factors. However, we believe that the associations between U-Cd and other renal biomarkers were caused by such co-excretion, as these associations were only seen with U-Cd (and not with K-Cd), or by chance, since many associations were assessed.

Co-excretion of cadmium and proteins

As previously described in the introduction, cadmium is bound to the protein metallothionein (MT), and circulates in blood plasma as a LMW Cd-MT complex. This complex is probably filtrated in the glomeruli and reabsorbed in the proximal renal tubules in the same way as other LMW proteins, such as A1M, B2M and RBP (21, 38, 125, 126). As discussed by Chaumont et al. (2012), the reabsorption in the proximal tubules probably occurs by the binding of LMW proteins, and also albumin, to two common receptors (megalin and cubilin) and subsequent endocytosis (61). The reabsorption of a protein is dependent on both its concentration and on its affinity to the receptor. Different mechanisms explaining the co-excretion of Cd and proteins have been proposed (60-62). One is the physiological variation in the proportion of LMW proteins reabsorbed in the proximal tubules, which would lead to a linear association between Cd and LMW proteins (61). Another suggested mechanism is that an increased concentration of albumin in the tubules leads to increased competition for the receptors, and that this may inhibit the reabsorption of the Cd-MT complex more than the reabsorption of other LMW proteins, because of the lower concentration of Cd-MT in tubular fluid (61). It has also been suggested that Cd might be bound to and co-excreted with albumin in urine (62). In addition, the excretion of both Cd and proteins in urine can be affected by changes in urinary flow rate (diuresis) and smoking (60-62). Further research is needed to elucidate the mechanism of co-excretion of Cd and proteins in urine.

Associations between cadmium and A1M

The association between K-Cd and U-A1M in our study seemed to be approximately linear, and with no obvious threshold. After stratifying for smoking, the association between K-Cd and U-A1M was seen also in never-smokers. This strengthens the assumption that cadmium is the cause of the increased A1M excretion, as smoking is a potential confounder regarding both cadmium and urinary protein excretion (127). Furthermore, an association was also seen between B-Cd and ON U-A1M/h in multivariate analysis.

An increased excretion of LMW proteins in urine following exposure to cadmium is not always clinically significant, but might indicate a risk for more severe renal damage in the future. In the present study, almost all U-A1M values were within the normal range.

A1M in urine has previously been considered mainly to be a marker of tubular dysfunction. However, it is possible that other mechanisms than decreased tubular reabsorption of proteins can affect U-A1M excretion. In a review article from 2014 Akerstrom and Gram presented A1M as a "housekeeping protein", suggesting that it acts as a protective antioxidant, continuously cleaning tissues from free radicals and oxidants, and repairing oxidative lesions (128). A1M is secreted from the liver, but also from most other epithelial cells, and is present in blood as well as intra- and extracellularly in all tissues. The expression of A1M is thought to be upregulated during oxidative stress, and after binding and neutralizing the free radicals and oxidants, these are delivered to the kidneys where they are degraded or excreted (128, 129). One possible mechanism explaining the association between cadmium and U-A1M could be that cadmium causes oxidative stress or tissue damage, which stimulates the production of A1M and leads to an increased excretion of A1M in urine. The presence of another mechanism than decreased tubular reabsorption could explain why no associations were found between K-Cd and the other renal biomarkers in our study (Paper III).

Differences between men and women

When we stratified the multivariate regression analyses for sex, the positive associations between K-Cd and U-A1M were found only in men (ON and 24-hour samples, per hour and creatinine-adjusted). No significant associations were found in women. The reason for this difference is unclear, but one possibility is that men are more susceptible to kidney damage. One indication of this is that men have a higher prevalence and incidence of renal disease, both diabetic and non-diabetic (130). Furthermore, the rate of

progression in renal disease has previously been thought to be higher in men than in premenopausal women, possibly explained by hormonal differences (130). However, in a meta-analysis from 2013, the authors found that the risk of progression to end stage renal disease was the same in men and women at a given eGFR and urinary albumin/creatinine ratio (131).

Associations with mGFR and eGFR

Associations were found between cadmium biomarkers and eGFR, estimated from cystatin C, but not with mGFR, in our study (Paper III). Estimated GFR was approximately 10% lower than mGFR, and the precision was much lower, indicated by the wide ranges. Previous research has also shown that eGFR might be imprecise and biased at normal or almost normal GFR (132-134). In the present study, mGFR was ≥90 mL/min in 78% of the subjects, and <80 mL/min only in two subjects. As mGFR is more reliable and the gold standard, our interpretation of the results is that there was no association between cadmium and GFR.

5.1.6 Urinary cadmium and BMD (Paper IV)

Significant negative correlations were found between continuous U-Cd and total body BMD, and sBMD for total hip, femoral neck, trochanter, and lumbar spine, in a cohort of elderly men in Gothenburg. In the multivariate regression analyses, the associations between continuous U-Cd and BMD were not significant, in a model including age, BMI, pack-years, and physical activity. However, when the covariate pack-years was not included, significant negative associations were found with total body BMD, and with total hip and trochanter sBMD. As discussed later, this might be the result of over-adjustment when smoking is included in the multiple regression models. In addition, total body BMD, and sBMD for all sites, was significantly lower in the fourth quartile of U-Cd compared to the first quartile, after adjustment for the same covariates (age, BMI, pack-years, and physical activity).

Previously, studies of the relationship between low-level cadmium exposure and BMD have been conducted mainly on women or on populations with higher exposure (76, 79, 80, 82, 83, 86, 89, 113, 114). Only a few studies have presented results for men separately. In two relatively small studies, no associations were seen in men (114, 135). In the first study, conducted on 199 men and 307 women in Belgium, Staessen et al. (1999) found significant negative associations between U-Cd (mean 8.7 nmol/day) and forearm BMD, but not in men (114). In the second study, Trzcinka-Ochocka et al. (2010) studied 170 women and 100 men living near a zinc-smelter in

Poland, but found no association between U-Cd (geometric mean 0.88 µg/g creatinine in men) and BMD (135). However, there are a few previous studies that suggest an association also in men. Alfvén et al. (2000) studied 520 men and 544 women in Sweden, living in areas with former environmental cadmium pollution from industries, or with occupational exposure to cadmium (mean U-Cd in men 0.38 and 2.1 nmol/mmol creatinine, respectively), and found negative associations between U-Cd and forearm BMD in men (83). Wu at al. (2010) studied >10,000 men and women in the United States, and found that odds ratios for the prevalence of osteopenia and osteoporosis increased with increasing U-Cd both in men and women (mean U-Cd in men 1.06 µg/g creatinine for smokers and 0.65 for non-smokers) (82). In a study of 83 men working in a radiator factory using cadmium-containing solder, Nawrot et al. (2010) found that U-Cd (geometric mean 1.0 µg/g creatinine) was associated with lower BMD in the distal forearm, higher U-Ca and an increased risk of osteoporosis (88). In a Korean study including 456 men, Kim et al. (2014) found a significantly higher prevalence of osteoporosis in men, but not women, in those with high U-Cd ≥5 µg/g creatinine (136). The results from our study provide further support for an association between low-level cadmium exposure and decreased BMD in the general population also in men.

5.1.7 Urinary cadmium and fractures (Paper IV)

A positive association was found between relatively low levels of cadmium in urine at baseline (year 2002-2004) and the risk of incident non-vertebral osteoporosis fractures, in a cohort of elderly Swedish men, both in 2009 and in 2013. The increased HRs were still statistically significant after adjustment for age, BMI, smoking and daily walking distance, comparing the highest quartile of U-Cd to the lowest. Most previous studies of low-level cadmium exposure and fracture risk have been conducted mainly on women, and only a few of the studies on men have used U-Cd to assess exposure. For example, Thomas et al. (2011) found positive associations between dietary Cd and the overall fracture rate in men, and Dahl et al. (2014) found that the risk of hip fractures in men was associated with the concentration of Cd in drinking water (75, 81). Staessen et al. (1999) found an association between U-Cd and fracture risk in women but not in men, which might be the result of the young age of the study participants and the short follow-up time (mean age 44 years, median follow-up time 6.6 years), resulting in only 44 fractures in a study population of 506 men and women (114). Alfvén et al. (2004) found an association between U-Cd and an increased risk of forearm fractures in those >50 years of age, in a study of 1021 Swedish men and women (112). Our results provide further support for

an association between low-level cadmium exposure and an increased risk of fragility fractures also in men, in the general population.

Cadmium was not associated with "other fractures", i.e. fractures that are not related to osteoporosis, in any of the statistical analyses in our study. This is interesting as well, as it further supports the hypothesis that cadmium increases the risk for osteoporotic fractures, but not other types of fractures.

In the present study, the covariates age, BMI, smoking, and physical activity were included in the main model (model 2), as they are considered to be important factors that affect the risk of fragility fractures (67, 72). Estimated GFR, SHBG, and falls during the last year were not included in the final model as the effect on the results was negligible.

As discussed previously (paragraph 1.4 and 5.1.3), many different mechanisms have been proposed for the effects of cadmium on bone, such as impaired absorption of calcium in the gut, decreased reabsorption of calcium in the renal tubules, influence on PTH or vitamin D activation, or a direct effect on the bone cells. BMD was not included in the main model in our study, as decreased BMD caused by cadmium is thought to be part of the mechanism behind the increased fracture risk. However, when BMD was included in the model (model 3), HRs were only slightly lower than in the main model. This could indicate that cadmium increases fracture risk also by affecting other factors than BMD, such as other determinants of bone quality or factors that increase the risk of falling.

5.2 Methodological considerations

Validity aspects in Paper I-III

In Paper I-III, the study population consisted of living kidney donors, recruited from the Department of Transplantation and Liver Surgery at Sahlgrenska University Hospital. The group was thus not randomly selected, but it consisted of both women and men, young and old, both smokers and non-smokers, and they were healthy enough to be accepted as kidney donors, with normal kidney function. Based on this, we believe that the donors may be representative of the healthy population in Sweden. Normally, it would hardly be ethical to take kidney biopsies from healthy, living persons, because of the risk of complications. Therefore, most of the previous knowledge on kidney metal concentrations comes from autopsy studies, and these cases have often died in accidents or following sudden disease (4-20, 34). Our study is thus more likely to reflect the kidney metal levels in the

(healthy) general population, as the autopsy studies may be based on a more selected underlying population. However, the kidney metal levels in our study agree well with the results from previous studies in Sweden (7, 9, 107).

Another advantage with the study population in Paper I-III, compared to autopsy studies, was the possibility to link first-hand information about exposure sources and background factors to the levels of heavy metals in the kidney. It was also possible to compare kidney metal levels with the metal concentrations in blood and urine, as well as concentrations of effect biomarkers, like LMW proteins in urine. There was also no risk of post-mortem changes, which can occur in autopsy studies. The 24-hour urine samples were collected after admission to the hospital, which is likely to have increased both the total number of 24-hour samples and the proportion of correctly collected samples. In total, seven 24-hour samples were excluded due to deviant or missing volumes, before the statistical analyses were made. Analyses of RBP and KIM in urine were performed at the department of Occupational and Environmental Medicine, University of Gothenburg, Sweden, with sufficient precision. The metal analyses will be discussed separately later in this subchapter. All other analyses in urine and serum were performed with standard methods at the Sahlgrenska University Hospital, Gothenburg, Sweden.

One limitation with the TINA study is the size of the study population (109 donors), resulting in limited power to detect true associations. As the cadmium levels were relatively low, it might also not be possible to use the results for populations with higher levels of exposure. Furthermore, as all participants were relatively healthy, conclusions cannot be drawn about the effects of the same cadmium levels in persons with medical conditions such as diabetes or hypertension, as they might be more susceptible to cadmium toxicity.

Validity aspects in Paper IV

In Paper IV, the study population consisted of 936 elderly men from Gothenburg, aged >70 years old. The high age of the participants (mean age 75 years at baseline) could be a limitation of the study, as the effect of cadmium probably differs between different age groups (112). However, this could also be a strength as cadmium is accumulated in the human body during the years, and the effects on bone might not appear until later in life. Furthermore, since elderly people have a higher incidence of fractures, the power to detect an association increases (63). The size of the cohort is relatively large, which is also a strength considering generalizability.

Fracture data is very trustworthy, as only X-ray verified fractures were included. There is a risk that we have lost a few cases with fractures that have occurred in other parts of the country or abroad, but those are probably very few considering the high age of the participants, and the fact that an X-ray control is made for most major fractures.

We chose to study the risk for fractures in relation to baseline cadmium both in 2009 and in 2013 (Paper IV). The reason for this was that we wanted to examine if there were any differences in risk after five and ten years, respectively. HRs were also generally higher at first follow-up in 2009 than in 2013. One possible explanation for this could be that the baseline data that was included in the multivariate analyses was not as relevant after 10 years as after 5 years.

A potential problem with the covariate pack-years in Paper IV is that in the questionnaire, the participants were only asked how many cigarettes they smoked per day on average, i.e. during all years they had smoked. There is a risk that they have underestimated or overestimated the average amount.

Metal analyses

One potential problem when metal concentrations are analyzed in biological samples is contamination with metals from the sampling material. In the TINA study (Paper I-III) all samples for metal analyses were collected using material free from cadmium, mercury and lead. Morning urine and 24-hour urine samples were collected in pre-washed polypropene bottles at the hospital, and aliquots for cadmium analysis were transferred to polypropene tubes, in order to avoid contamination with cadmium. Blood samples for analysis of Cd were taken in Venoject II glass tubes free from cadmium, lead, and mercury, and frozen for later analysis. The part of the kidney biopsy that was intended for metal analysis was transferred into a pre-acid washed glass tube and also frozen for later analysis. In the MrOS study (Paper IV), urine samples from the baseline examination in 2002-2004 were frozen, and analyzed for cadmium in 2012. Contamination with cadmium could be a potential problem in this study as the samples were not originally collected for metal analyses, and one man was excluded due to very high urinary cadmium though he had only smoked for two years. However, in the other subjects the levels of cadmium in urine were in the expected range.

Molybdenum oxide-based interference is also a potential problem when cadmium is analyzed in urine samples with ICP-MS. Molybdenum is naturally occurring in urine, and molybdenum oxide can be formed from molybdenum during the analysis, and interfere with cadmium. Originally,

half of the urine samples in the TINA study were analyzed with a method not correcting for molybdenum oxide-based interference. However, all samples were reanalyzed in 2012 with an appropriate method, and we only used the U-Cd values from the reanalysis in the present study. Furthermore, there was a good agreement between the original and the new U-Cd concentrations for samples that had been corrected for molybdenum oxide-based interference also in the first analysis, as previously described by Akerstrom et al. (95).

As we studied populations with low-level exposure, the concentrations of metals in kidney, blood and urine were generally low. This was as we expected, but those low levels place high demands on the accuracy of the analyses. As for the kidney metals (Paper I-III), which were analyzed in four different rounds, external quality control samples were analyzed six times in each round, and the results were satisfactory compared to the target values. No K-Cd values were below LOD, but 32 K-Hg values and eleven K-Pb values were below LOD. For K-Hg and K-Pb <0.01 µg/g ww, the value 0.01 was used in the statistical calculations. For concentrations >0.01 µg/g, the estimate from the chemical analyses was used. According to Hornung and Reed (1990), LOD/2 can be used for values below LOD when data are highly skewed, otherwise LOD/√2 can be used (96). In Paper II, U-Cd values below LOD were replaced by LOD/√2, whereas for B-Cd, LOD/2 was used, according to the data distribution. In the MrOS cohort (Paper IV) five of 936 U-Cd values were below LOD (LOD 0.05 µg/L). For these values we used the estimate from the analysis (0.01-0.03 µg/L), as they were very close to the LOD. In addition, the methods described by Hornung and Reed would give values in the same range (96). Three quality control samples were used for U-Cd in the MrOS cohort, and the results agreed well with the recommended values.

Confounders

The independent variables that were included in the multivariate models were either considered to be true confounders, or well-known risk/protective factors regarding the outcome or the dependent variable.

Age is considered a confounder regarding cadmium and bone effects, as both the body burden of cadmium and fracture risk increase with age, and BMD decreases with age (34, 67). In addition, kidney function decreases with age (52). We therefore adjusted for age in all multivariate models.

As urinary flow rate affects the excretion of LMW proteins in urine, and also the excretion of Cd, this variable was included in one of the multivariate

models in Paper III. Urinary flow rate was also included in the model in Paper II, with U-Ca as the dependent variable, after backward elimination of non-significant variables. In Paper II, we also adjusted for menopause. Menopause is a possible confounder as it can affect both the absorption of cadmium and bone metabolism. One limitation is that we had no information about the actual age of menopause. Instead we constructed a dummy variable, assuming menopause at age >51 years.

Smoking is a well-known risk factor for osteoporosis and fractures (137). However, cumulative smoking is also the main source of cadmium in smokers. Consequently, there is a risk of over-adjustment when we include pack-years as a covariate in the multivariate model. In Paper IV, HRs of incident fractures were generally higher when pack-years was replaced by the covariate current smoking, probably because pack-years is a better measure of cumulative smoking. U-Cd and pack-years were rather highly correlated (r_s=0.53, p<0.0001). In smokers, including pack-years as a covariate probably could mean that the effect of cadmium from smoking partly vanishes, and that most of the detected effect on fracture risk comes from cadmium in the diet.

It is possible that the effect of smoking on bone is in part mediated through cadmium (138). However, cigarette smoke contains a wide range of chemicals, and many different mechanisms behind the effect of smoking on bone have been proposed, such as impaired absorption or altered metabolism of calcium and vitamin D, lower BMI, early menopause, lower circulating levels of estradiol, oxidative stress, or a direct effect on osteoblasts and osteoclasts (137).

6 CONCLUSIONS

- Kidney cadmium levels (K-Cd) in Sweden have decreased since the 1970s, probably due to decreased smoking, as shown in the present study on living, healthy kidney donors. However, the intake of cadmium via diet seems unchanged, as the levels in never-smokers were very similar to those found in the 1970s.

- Female never-smokers had higher K-Cd levels than male never-smokers, probably caused by increased gastro-intestinal absorption of cadmium in food due to low iron stores in many women, and possibly also because women in general have smaller kidneys.

- Kidney mercury levels were associated with the number of dental amalgam surfaces, but not with fish consumption.

- Kidney lead levels were lower than previously reported from autopsy studies, probably due to decreased exposure during several decades.

- Women, but not men, with high K-Cd (above the median) had significantly higher excretion of calcium in urine.

- The excretion of the LMW protein alpha-1-microglobulin in urine was positively associated with the cadmium levels in kidney, blood and urine. Other biomarkers of renal function, and GFR, were not associated with K-Cd. This could indicate that alpha-1-microglobulin is a sensitive biomarker for early effects of cadmium. Associations between urinary cadmium and other biomarkers of renal function were probably caused by co-excretion or chance.

- In a cohort of elderly Swedish men, higher levels of cadmium in urine were associated with lower BMD, and an increased risk of non-vertebral osteoporosis fractures.

The results from this thesis indicate that low-level exposure to cadmium, as found in the general population in Sweden, has effects on both kidney and bone.

7 FUTURE PERSPECTIVES

It is clear that exposure to cadmium will continue to be an important issue also in Sweden in the future, and it is important to try to minimize the spread of cadmium in the environment by reducing the emissions of cadmium to the air, also from other European countries, thereby reducing the atmospheric deposition of cadmium. It is also important to reduce the use of cadmium-containing fertilizers, improve the recycling of metals and nickel-cadmium batteries, and reduce the use of toxic heavy metals in general.

The research presented in this thesis contributes to increased knowledge on the levels of cadmium, mercury and lead in the Swedish population, and the potential health effects related to low-level cadmium exposure, particularly associations with renal biomarkers, bone mineral density and fractures. However, further research is needed to confirm the results, and to elucidate the mechanisms behind the effects on bone mineral density and fracture risk. A future study could include a larger cohort, with longer follow-up time and repeated cadmium samples. Concerning the renal effects, a study of associations between cadmium levels in the kidney and histopathological findings could contribute to increased knowledge. Furthermore, more studies are needed to examine the relationship between cadmium and cardiovascular disease. An important question is whether there is a "safe" level of cadmium exposure, and if this level differs between different populations and age groups.

ACKNOWLEDGEMENT

I would like to thank everyone who contributed to this thesis, especially:

My supervisors Lars Barregård and Gerd Sällsten, for encouraging me, helping me, sometimes pressing me, and believing in me. I wish I could become a walking encyclopedia too.

My co-authors for support and advice.

All members of my research group (Miljömedicin och toxikologi) for intelligent comments and discussions, and for being nice and friendly.

Göteborgs Läkaresällskap for financial support.

Jag vill också tacka:

Alla trevliga arbetskamrater på Arbets- och miljömedicin, som kan lite om allt och mycket om lite, ibland även mycket om mycket. Det finns alltid någon att fråga om man undrar något, vare sig om det handlar om arbetet eller helt andra saker.

Gunnel Garsell, för all vänlig och praktisk hjälp under åren. Cecilia Andreasson, för hjälp med diverse praktiska saker inför disputationen. Leo, Sandra, Maria och Mia, för tips och hjälp när det har närmat sig dagen D.

Gänget från läkarutbildningen med respektive: Helena, Michael, Erika, Henrik, Lisa, Daniel och Ola. Tack för att ni är så roliga, smarta och lojala.

Gamla vännerna från Vargön och Vänersborg, för att ni fortfarande finns kvar trots att vi ses så sällan.

Min familj, som hjälper mig att fokusera på det riktiga livet när jag kommer hem.

Mina föräldrar, för att ni alltid har låtit mig göra som jag vill.

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