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Methods and Techniques

A protocol for the prospective study of urinary cadmium with risk of fracture, bone loss, and muscle loss

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Abstract

Cadmium (Cd) is a heavy metal and natural element found in soil and crops with increasing concentrations linked to phosphate fertilizers and sewage sludge applied to crop lands. A large fraction of older US men and woman have documented Cd exposure. Cd exposure has proven health concerns such as risk of lung cancer from inhalation and impaired renal function; however, growing evidence suggests it also influences bone and muscle health. Given that low levels of Cd could affect bone and muscle, we have designed prospective studies using the two largest and most detailed US studies of bone health in older men and women: the Osteoporotic Fractures in Men Study and the Study of Osteoporotic Fractures. We are investigating the association of urinary cadmium (U-Cd), as a surrogate for long–term Cd exposure, with bone and muscle health. Building off suggestive evidence from mechanistic and cross–sectional studies, this will be the first well–powered prospective study of incident fracture outcomes, bone loss, and muscle loss in relation to U-Cd, an established biomarker of long–term Cd exposure. The following is a proposed protocol for the intended study; if successful, the proposed studies could be influential in directing future US policy to decrease Cd exposure in the US population similar to recent policies adopted by the European Union to limit Cd in fertilizers.

Keywords: aging, osteoporosis, biochemical markers of bone turnover, statistical methods, fracture risk assessment

Introduction

Cadmium (Cd) is a heavy metal and natural element found in soil, with increasing concentrations linked to phosphate fertilizers and sewage sludge applied to croplands. According to the US Food and Drug Administration (FDA), the total dietary background ingestion of Cd among Americans is 0.26 μg/kg/d.[1] Additionally, Cd levels detected in human bones have increased 10-fold since preindustrial times.[2] Besides diet, smoking is another primary source of Cd exposure. Cd has been recognized as an occupational health hazard for decades, notably as a risk factor for lung cancer and impaired renal function.[2–6] Among other health concerns, Cd has been implicated as a possible risk factor for osteoporosis because of its known accumulation in bone tissue.

The international reference standard of osteoporosis defined by the World Health Organization (WHO) is bone mineral density (BMD) that lies 2.5 standard deviations or more below the mean for young healthy women (a *T*-score of <-2.5 SD) at the unilateral femoral neck via dual–energy X–ray absorptiometry (DXA).[7] Globally, osteoporotic fractures are a major cause of morbidity and mortality with the risk of osteoporosis greatest in older women. The 10–yr absolute risk of fracture for those aged 75-84 with osteoporosis is 24% in women and 14% in men; because of population aging, economic and healthcare strain is only expected to increase.[8] Although the FDA has approved some effective medications to mitigate fracture risk, other strategies should be explored because of the significance of the problem. Notably, it has long been known that exposure to high levels of Cd is associated with osteomalacia, osteoporosis and fractures because of its adverse effects on the kidneys.[5,9,10] However, recent evidence suggests that low–level Cd exposure may have adverse effects on bone that are independent of kidney disease.[11-15] For example, mechanistic studies from bone organ and cell culture systems suggest that Cd increases bone remodeling and reduces bone mass independent of renal effects and has been documented to accumulate in osteocytes, the periosteum, and bone marrow.[16-18]

In 2008, we were the first group to show associations with osteoporosis, defined by either femur neck BMD < 0.56 g/cm^2 or total hip BMD < 0.64 g/cm^2, consistent with the WHO international reference standard[7], from creatinine–adjusted

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urinary cadmium (U-Cd_{cr}) at low levels common in the population (0.5-1 ug/g U-Cd_{cr}).[19] Since then, a few substantial epidemiologic studies have reported associations at similar levels of U-Cd_{cr}. Among 2688 Swedish women, the odds ratios from a cross–sectional study were 2.45 (95% confidence interval (CI): 1.51-3.97) for osteoporosis at the femoral neck and 1.97 (95% CI: 1.24-3.14) for osteoporosis at the lumbar spine among those with U-$Cd_{cr} \geq 0.75$ μg/g (compared with <0.50 μg/g).[20] Among 936 men studied cross–sectionally, standardized BMD was lower by 0.05 g/cm^2 for hip and femoral neck, and by 0.07 g/cm^2 for trochanter between first (mean 0.14 μg/g) and fourth quartile (mean 0.67 μg/g) U-$Cd_{cr.}$[21] To our knowledge, the above study was the first prospective study of urinary cadmium (U-Cd) and fracture. Among all participants, OR = 1.4 (95% CI: 0.9, 2.2) for all fractures (N = 229), OR = 1.6 (95% CI: 1.0, 2.7) for osteoporotic fractures (N = 173); and among never smokers, OR = 1.5 (95% CI: 0.6, 3.6) for all fractures (N = 76), OR = 1.9 (95% CI: 0.7, 5.1) for osteoporotic fractures (N = 56) comparing upper quartile vs lowest quartile U-Cd_{cr} (mean 0.67 vs 0.14 μg/g).[21] A recent mediation analysis using the same the Osteoporotic Fractures in Men (MrOS) Sweden cohort suggested 59% of smoking–related osteoporosis, and 20% of smoking–related osteoporotic fracture is mediated via U-Cd.[22] These results were suggestive but underpowered leaving a demand for further prospective cohort studies of the link between Cd and bone health.

There also is emerging evidence that Cd may be associated with muscle loss. One cross–sectional study from 2016 involving 704 participants (344 males, 360 females), after adjustment for BMI and waist circumference, reported a decreased appendicular skeletal muscle mass index (< 5.4 kg/m^2 in females and < 7.0 kg/m^2 in males) was associated with increasing blood Cd levels for both males (P = .010, OR = 1.42) and females (P = <0.001, OR = 4.41).[23] More recently, a cross–sectional study in National Health and Nutrition Examination Survey of 4333 participants from 2020 showed that both blood Cd and urine Cd had an inverse dose–response relationship with grip strength, a well–known biomarker of negative health outcomes in older adults.[24] This study showed a 1.93 kg reduction in combined grip strength in highest quartile (fourth quartile compared with first) blood Cd concentration (P < .001) and a 3.24 kg reduction in the highest quartile urine Cd concentrations (P < .001).[24] Although both cross–sectional studies suggest that Cd is associated with loss of muscle mass and function, longitudinal investigation is warranted.

In light of growing evidence that low levels of Cd could affect bone and muscle, we have designed prospective studies using the two largest and most detailed US studies of bone health in older men and women (MrOS Study and the Study of Osteoporotic Fractures (SOF)) to investigate the association of U-Cd, as a surrogate for long–term Cd exposure, and bone and muscle health. The following objectives will be investigated:

- Objective 1: using a case–cohort study design, we will determine the association between U-Cd and incident fractures separately in older men and women.
- Objective 2: evaluate the prospective association between U-Cd and rate of loss of total hip BMD separately in men and women.
- Objective 3: provide insight into the cellular and structural mechanisms by which Cd may adversely affect bone such as the association between U-Cd and markers of bone formation (PINP), bone resorption (CTX), and bone structure (high–resolution peripheral quantitative–computed tomography (HR-pQCT)).
- Objective 4: evaluate the association of U-Cd and grip strength, gait speed, and muscle mass (objective 4) separately in men and women.

We hypothesize that increased Cd will be associated with increased fracture risk (objective 1), increased bone loss (objective 2), decreased bone strength/increased bone turnover (objective 3), and increased muscle loss (objective 4).

Materials and methods

Study sample and participants

We are leveraging existing data and samples from the MrOS and the SOF cohorts, which are the world's premier studies of skeletal aging in men and woman, respectively. Key features of these cohorts include their size, statistical power, long follow-up, and biospecimen reserves, which together serve as a strong opportunity to delineate associations of Cd with bone and muscle. In both cohorts, rich covariate data were collected on many factors, including diet, smoking, demographics, and risk factors. Dual X–ray absorptiometry (DXA) scans, grip strength, gait speed, and repeated chair stands were collected at each visit, and incident fractures radiographically confirmed and adjudicated every 4 mo for the study duration.

The MrOS prospective cohort study follows ambulatory men ≥65 yr of age at six US clinics (Birmingham, AL; Minneapolis, MN; Palo Alto, CA; Pittsburgh, PA; Portland, OR; San Diego, CA). For detailed information see https://mrosonline.ucsf.edu[25]; 5994 men were enrolled from March 2000 through April 2002. Second–void morning urine samples and serum following overnight fasting were collected at baseline and stored at −80°C without thawing. All men were subsequently invited to take part in multiple follow–up visits with >95% rates of participation among survivors. In our investigation, we will include men from March 2000 as our starting follow up population.

The SOF cohort is a multicenter prospective cohort study of primarily Caucasian, community–dwelling women aged ≥ 65 yr from four geographic areas (Portland, OR; Minneapolis, MN; Pittsburgh, PA; Baltimore, MD) from 1986 to 1988. The 9704 participants comprising the original cohort were subsequently invited to take part in nine principal follow–up visits with >95% rates of participation among survivors. A detailed study description was published previously.[26,27] Aggregate morning urine samples collected over a 2–h window as well as serum samples, following overnight fasting, were collected and stored at −80°C. We will include women and their urine samples from SOF visit 6, which included the recruitment of an African American cohort (1997-1998) as our starting population for follow-up.

Validity of urine Cd as a biomarker for long–term exposure

Traditionally, a biomarker indicative of long–term exposure to an environmental contaminant is rare. For this investigation, U-Cd was chosen as the primary biomarker for exposure in

both males and females because of its previous validation as a long–term biomarker. In short, the synthesis of metallothionein (which has anti toxin effects on Cd) can be induced by Cd.[28,29] The Cd–metallothionein complex is efficiently cleared from blood plasma by glomerular filtration and reabsorbed into the proximal tubules of the kidney leading to Cd accumulation in the kidney with a half-life of 10-30 yr.[3,30] In the general (nonoccupationally exposed) population, U-Cd is considered a biomarker for long–term Cd burden because U-Cd levels are proportional to the level of Cd that has bioaccumulated in the kidney (primary target organ).[2] Furthermore, we independently reviewed the U-Cd biomarker[31] and reported an intraclass correlation coefficient (ICC) for creatinine corrected U-Cd (U-Cd_{cr}) of 0.66-0.81 in studies using interference correction strategies in the analysis.[32-36] There was no difference whether the samples were spot urines, first morning voids, or from different time intervals ranging from weeks to months to 3 yr; however, these studies did not include older persons. Our study in these cohorts confirms the excellent replicability of U-Cd_{cr}, with ICC = 0.81 among 19 women sampled 4 yr apart from SOF and ICC = 0.74 among 39 men sampled 6 yr apart from MrOS.[37] The weight of evidence suggests that a single spot urine sample is a stable, representative estimate of decades–long Cd exposure. Lastly, it is important to note that established determinants of U-Cd levels include age, gender, smoking status, dietary habits, and occupation all of which we will account for in our analysis.[30,38-41]

Urine analyses

Using previously collected urine samples from the MrOS and SOF studies, laboratory analyses for metals will be carried out by RTI International's Trace Inorganics Laboratory in RTP, NC. While our primary hypotheses concern U-Cd levels, these urine samples are valuable and as a result, we are electing to analyze additional metals as well, including antimony, arsenic, barium, beryllium, cesium, cobalt, lead, manganese, mercury, molybdenum, platinum, selenium, strontium, thallium, tin, tungsten, uranium, and zinc. An iCAP Q quadrupole inductively coupled plasma–mass spectrometer (Thermo, Waltham, MA) will be employed for determination of trace metals in urine, equipped with a collision cell to mitigate the impact of polyatomic interferences along with previously validated protocols.

Urinary cotinine will also be quantified using commercially available, solid phase–competitive ELISA kits (eg, Abnova Corporation: KA0930). Cotinine is commonly known as a strong biomarker of tobacco smoke exposure and therefore is useful in identifying smokers.

Urinary concentrations of contaminants are highly influenced by the degree of urinary dilution, thus adjusting values for dilution is critical.[42] Urinary creatinine concentrations are most typically measured. In our study, these will be quantified using a Caymen Chemicals Creatinine Assay Kit No. 500701 (Cayman Chemicals, Ann Arbor, MI, USA) with UV–VIS measurement at 500 nm employing a Beckman Coulter DU800 UV/VIS Spectrometer (Beckman Instruments Inc., Brea, CA, USA). We will also measure osmolality that is strongly correlated ($\rho = .75$) with specific gravity, making it useful as a control for urine density.[43,44] We will quantitate osmolality by freezing point depression using an Osmometer Model 3320 (Advanced Instruments Inc.).

Control for urinary density

Traditionally, epidemiologists have adjusted for creatinine by dividing the analyte concentration by the urinary creatinine concentration. However, Barr et al.[45] suggested that adjustment should be done statistically, by including creatinine concentrations as a separate variable in the statistical model. While the choice of standardization method remains debated, in our primary analysis, we will adjust within the Cd measure (μg Cd/g cr) because of the high ICC of the creatinine standardized value, and secondarily adjust for creatinine statistically. In our previous experience, results were not materially different across these methods of creatinine adjustment.[46] We will also secondarily use an approach that involves a covariate–adjusted Cd/creatinine ratio[47] and we will further compare results, as well as carry out the same set of analyses using osmolality in place of creatinine.

Objectives: sample size, outcomes, and statistical analysis

Objective 1: using a case–cohort study design, we will determine the association between U-Cd and incident fractures separately in older men and women

The primary outcome being studied is defined by all fractures reported through December 2021 (estimated to be 1321 cases MrOS, 1578 cases SOF). Secondary outcomes will include major osteoporotic fractures defined as fractures at the wrist, upper arm, hip, and clinical spine; that is, sites associated with low BMD (estimated to be 676 cases MrOS, 946 cases SOF) and hip fractures separately (estimated 310 cases MrOS, 581 cases SOF). We will select incident cases of fracture in men and women, and compare randomly selected subcohorts of 1500 MrOS men and 1500 SOF women. Incident clinical vertebral and non–vertebral fractures were identified from participant (or, if deceased, participant's contacts) responses to every 4–mo mail or phone queries about new fractures with more than a 98% and 99% completion rate among active surviving participants through follow–up times of 13 and 20 yr, respectively, for SOF and MrOS cohorts. Incidental vertebral fracture cases will be identified by the following criteria: (1) symptoms suggestive of vertebral fracture that prompted participant to seek medical attention and (2) a community imaging study (eg, radiograph, computed tomography) centrally reviewed by a masked study physician that showed a ≥ 1 increase in semiquantitative grade compared with the same thoracic or lumbar vertebrae on baseline study radiograph.[48-50] Cd, creatinine, osmolality, and cotinine will be analyzed in urine; and creatinine will be analyzed in serum.

Statistical analysis will be carried out according to the case–cohort design, and the unweighted case–cohort approach used for analyses.[51] Separate analyses for each fracture endpoint (all, hip, vertebral, and major osteoporotic) will be conducted in each cohort. For each analysis, we will calculate the person-years from baseline to the date of fracture event, the date of death because of causes other than fracture event, or the date of administrative censoring, whichever occurred first. We will use Cox proportional hazards models (PHREG procedure of SAS 9.4 (SAS Institute, Cary, NC)) to estimate hazard ratios as a measure of the strength of association between U-Cd and incident fracture. Age will serve as the time axis, and analyses will be corrected for delayed entry. We will also calculate two–sided 95% CIs and *P*-values on the basis of robust estimates of the variance–covariance matrix[52] and

Wald test statistic for regression parameters in Cox regression models. U-Cd will be split into quartiles to test for possible threshold or nonlinear effects as the primary analysis, and as a continuous variable, natural log-transformed to assess dose–response trend in a secondary analysis. We will investigate the shape of the dose–response curve using splines, exploring sensitivity to number of knots using visual inspection and formal testing (eg, using the Akaike information criterion) to select best fit model.[53] Additionally, the model will be adjusted for confounders via a list of covariates based on prior knowledge and observed associations between each covariate and both the exposure and outcome. Factors considered intermediaries on the causal pathway (eg, mediators) will not be considered as confounders.

Objective 2: evaluate the prospective association between U-Cd and rate of loss of total hip BMD separately in men and women

The primary outcome for objective 2 is total hip BMD as recommended by the International Society of Clinical Densitometry.[54] Generally, the right hip will be used for BMD analysis unless there was a fracture, implant, hardware, or other problem preventing proper measurement of the right hip, in which circumstance the left hip will be used for analysis. Secondary outcomes include femoral neck and lumbar spine BMD. In MrOS, DXA scans and urine were collected at baseline in 2000-2002, with follow–up DXA in 2005-2006, 2007-2009, and 2014-2016; at least one follow–up measure is available in 78% of the participants. In SOF, DXA and urine were collected in 1997-1998, with follow–up DXA in 1999, and 2002-2004; at least one follow–up measure is available in 59% of the participants. These subcohorts are representative of the full cohorts and therefore have findings generalizable to the full cohorts with statistical adjustment; they will serve as the sample for the primary analysis. The analysis will also be repeated among fracture cases to assess whether the relationship between Cd and BMD differs among fracture cases.

In both cohorts, return for follow–up visits was associated with self–reported good health at earlier visit (85.0% vs 77.3% in SOF; 89.5% vs. 72.8% in MrOS) and younger age (78.3 vs 81.0 in SOF; 72.9 vs 77.0 in MrOS). We can statistically adjust for these factors, and will investigate associations with U-Cd to assess whether any of these selection factors are potential confounders. To further address potential bias from missingness, we will conduct a sensitivity analysis limited to a subset of participants in the cohorts who have similar characteristics to those in the full cohort.

We will examine change in bone loss over time using individual growth models via a mixed modeling approach (SAS PROC MIXED). Mixed models are flexible in dealing with varying lengths of time between observations and missing data. Time from baseline (in months) will be entered as a predictor value, along with baseline U-Cd. The primary analysis will be carried out using U-Cd quartiles, whereas the secondary analysis will be carried out using a continuous U-Cd variable following the methodology from objective 1. To assess whether U-Cd is associated with increased rate of bone loss, we will test for an interaction between time and U-Cd, with a positive term indicating increased bone loss for higher U-Cd respondents. Time will also be included in the random part of the model, allowing for individual differences in rate of bone loss. The above approach assumes a heteroskedastic compound symmetric error covariance matrix; however, we will also test whether a more complex structure (eg, autoregressive) is indicated by comparing goodness of fit. Similar methods will be used to adjust for confounding variables as used in objective 1.

Objective 3: provide insight into the cellular and structural mechanisms by which Cd may adversely affect bone such as the association between U-Cd and markers of bone formation (PINP), bone resorption (CTX), and bone structure (HR-pQCT)

A study of CTX and PINP will be carried out in MrOS and SOF. PINP and CTX were chosen as the primary outcome measures via recommendations from the 2010 the International Osteoporosis Foundation (IOF) Working Group on Bone Marker Standards and also the National Bone Health Alliance in 2012.[55,56] In MrOS, baseline serum data for CTX and PINP are already available in 1008 participants selected at random from the entire MrOS cohort[57]; all of these individuals are included in our subcohort here. In SOF, we will be conducting serum analyses for PINP and CTX in 996 women in the subcohort who have serum available, and urine Cd will be measured from specimens collected at the same visit.

A study of bone structure will leverage HR-pQCT data collected in 2014-2016 in the MrOS cohort. We have HR-pQCT data on 460 members of the subcohort[48] and will measure Cd, metals, creatinine, and osmolality in urine collected at that visit because ~15 yr have passed between baseline urine collection and HR-pQCT measurement. A priori we are selecting finite element analysis (FEA) estimated failure loads for distal radius, diaphyseal tibia, and distal tibia as our primary HR-pQCT variables. Our secondary variables for each of these skeletal sites are total vBMD, total bone area, trabecular vBMD, trabecular bone area, trabecular thickness, trabecular number, cortical vBMD, cortical bone area, cortical thickness, and cortical porosity.

Generalized linear regression will be adopted for these studies using continuous PINP, CTX, and FEA as the dependent variables. U-Cd_{cr} (μg Cd/g cr) will be the independent variable and the analyses will be conducted separately in the two cohorts. The dependent variable will be log-transformed if necessary to satisfy regression diagnostic tests. For the HR-pQCT data, we will compare the cross–sectional relationship between U-Cd and FEA, and longitudinally between U-baseline Cd and FEA.

Objective 4: evaluate the association of U-Cd and grip strength, gait speed, and muscle mass separately in men and women

The primary outcomes are grip strength and gait speed, measured longitudinally in MrOS and SOF and independently shown to predict falls and recommended as measures of sarcopenia by the Sarcopenia Definitions and Outcomes Consortium.[19] Repeated chair stands will serve as the secondary outcome. In addition, D-3 creatinine (D3Cr) muscle mass will be analyzed as a primary outcome but is only available in MrOS in limited capacity.

In the MrOS subcohort, grip strength, gait speed, and repeated chair stands were collected at baseline in 2000-2002, with follow-up in 2005-2006, 2007-2009, and 2014-2016 and at least one follow–up measure is available in 78% of the

participants. In SOF, these measures were collected in 1997-1998, with follow-up in 1999, and 2002-2004 and at least one follow–up measure is available in 59% of the participants.

The statistical analysis plan mirrors objective 2 with different outcomes here such as grip strength, gait speed, and repeated chair stands. The statistical plan for analysis of D3Cr muscle mass will mirror that described in objective 3. Similar methods will be used to adjust for confounding variables as described earlier.

Sensitivity analysis: serum creatinine

Urine biomarkers (eg, U-Cd) are less stable when the kidneys start to fail. Therefore, sensitivity analyses will be run wherein we exclude those with estimated glomerular filtration rate (eGFR) < 60 from the epidemiologic analysis. Serum creatinine will be measured and used to calculate an eGFR using the Chronic Kidney Disease Epidemiology Collaboration equation.[58] Serum creatinine will be measured using a standard colorimetric–based assay manufactured by Arbor Assays (Ann Arbor, Michigan) at OHSU. Serum creatinine has already been measured in MrOS men and most women from SOF. Another 1360 SOF participants will be measured for serum creatinine by the OHSU Research Assay Core Laboratory.

Exploratory objectives

In addition to the proposed primary objectives, exploratory studies will also be conducted because of data availability. In short, the exploratory objectives are as follows; first, as a natural extension of objectives 1-3, we will examine the extent to which Cd's association with bone outcomes is mediated via bone turnover markers. For each potential mediator (CTX, PINP in men and women), we will confirm prospective associations between baseline Cd and the potential mediator of interest using unadjusted and adjusted linear regression models. Second, based on available data in MrOS, we will explore the relationship between U-Cd and estradiol. By leveraging bioavailable estradiol (as well as testosterone, and sex hormone binding globulin) measured at baseline in the full 1500 members of the MrOS subcohort, we hope to facilitate understanding of the mechanisms by which Cd impacts bone in men. Lastly, we will explore whether mixtures of metals and nutrients are associated with fracture, bone loss, and muscle loss using Bayesian kernel machine regression.

Discussion

Building upon suggestive evidence from mechanistic and cross–sectional studies, this will be the first well–powered prospective study of incident fracture outcomes, bone loss, and muscle loss in relation to U-Cd, an established biomarker of long–term Cd exposure. We are leveraging the two largest and most detailed studies of bone and muscle health in men and women with up to 20 yr of follow–up subsequent to collection of urine samples. The bone turnover markers and mediation analysis will help us understand cellular and structural mechanisms by which Cd may adversely affect bone. In addition, since Cd may also impact the nervous system,[59] which can contribute to falls, fractures, and muscle loss, we will attempt to disentangle the nervous system effects, for example, by investigating whether the magnitude of effect on grip strength differs from that on muscle mass.

A large fraction of older US men and women have documented Cd exposure. Because of recent policies adopted by the European Union to limit Cd in fertilizers, these studies could be pivotal in directing future US policy to decrease Cd exposure to the US population. Establishing Cd as a risk factor of bone and muscle health promises to have major implications for the prevention and control of bone and muscle disease.

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Conflicts of interest

The authors state that they have no known conflicts of interest to declare that could appear to influence or alter the work put forth in this study.

Data availability

No new data were generated or analyzed in support of this work.

Ethics

Stony Brook University IRB Office of Research Compliance approved this study and declared it to be exempt. The parent studies (MrOS and SOF) recruited participants with IRB–approved informed consent. No new data were generated or analyzed in support of this work.

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