The threshold level of urinary cadmium associated with increased urinary excretion of retinol-binding protein and β_2-microglobulin: a re-assessment in a large cohort of nickel-cadmium battery workers

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Accepted 23 June 2010
Published Online First 8 October 2010

ABSTRACT

Objective To evaluate the threshold value of urinary cadmium (CdU) for renal dysfunction on the basis of relationships unconfounded by protein degradation, diuresis and the renal effects associated with chronic smoking.

Methods We studied 599 workers (451 men, mean age 45.4 years) who were employed in four nickel-cadmium battery plants for 18.8 years on average. After adjustment for covariates by multiple regression, the CdU threshold values for increased concentrations of retinol-binding protein (RBPU) and b_2-microglobulin (b_2-mU) were assessed by logistic regression and benchmark dose analyses using as referents workers with CdU<1 μg/g creatinine.

Results Relationships between urinary proteins and CdU (μg/g creatinine) were influenced by sex, age, diuresis and especially smoking. When considering all workers, odds for abnormal RBPU and b_2-mU were significantly increased from CdU of 6-10 and >10, respectively. The benchmark dose (BMD5) and the benchmark dose lower limit (BMDL5) for a 5% excess in the background prevalence of abnormal RBPU and b_2-mU were estimated at 5.1/3.0 and 9.6/5.9. When excluding ever smokers, odds for abnormal RBPU and b_2-mU were both increased only among workers with CdU>10 (OR, 21.8, 95% CI, 6.4-74.4 and OR, 15.1, 95% CI, 3.6-63.1, respectively). In never smokers, these BMD5/BMDL5 of CdU were estimated at 12.6/6.6 and 12.2/5.5 while in ever smokers they were 6.2/4.9 and 4.3/3.5.

Conclusions On the basis of associations undistorted by smoking and adjusted for covariates, the BMDL5 of CdU for low-molecular-weight proteinuria induced by occupational exposure to Cd can be reliably estimated between 5.5 and 6.6 μg/g creatinine.

What this paper adds

- Studies evaluating the threshold levels of urinary cadmium (CdU) for increased excretion of low-molecular-weight proteins have so far regarded smoking as merely an additional source of cadmium without taking into account the detrimental effects of tobacco smoking on the kidneys.
- This study based on a large cohort of nickel-cadmium battery plant workers, shows that the renal effects of chronic smoking substantially distort the dose–effect/response relationships between the urinary excretion of low-molecular-weight proteins and that of cadmium.
- In never smokers, the CdU threshold values for abnormal retinol-binding protein and β_2-microglobulin and their lower CI limits were estimated at 12.6/6.6 and 12.2/5.5 μg/g creatinine, respectively.
- In ever smokers, these CdU thresholds were two to three times lower, reflecting the renal effects of both cadmium and chronic smoking.

INTRODUCTION

Cadmium (Cd), a by-product of zinc production, is one of the most toxic metals to which man can be exposed at work or in the environment.[1] Cd is primarily toxic to the proximal tubular cells where it selectively accumulates over time and may cause irreversible damage. The earliest sign of tubular dysfunction induced by chronic Cd poisoning is increased urinary excretion of low-molecular-weight (LMW) proteins (molecular weight <40 kD) such as β_2-microglobulin (β_2-mU) and retinol-binding protein (RBPU).[2] Studies conducted in the 1980s on active workers in the Cd industry demonstrated that this LMW proteinuria, also called tubular proteinuria, is likely to occur in approximately 10% of workers when the Cd concentration in kidney cortex exceeds approximately 200 ppm (μg/g wet weight of renal cortex).[1 3 4] These studies have also shown that before renal dysfunction develops, the amount of Cd stored in the kidneys can be assessed non-invasively by measuring the concentration of the metal in urine (CdU).[1] On the basis of the relationship between Cd concentrations in the urine and kidney cortex in workers with no renal dysfunction, the CdU value corresponding to the critical level of 200 ppm in kidney cortex was estimated at 10 μg/g creatinine,[1 3] a value in concordance with that derived from the relationships between β_2-mU and CdU.[5 6] The occupational exposure limit of 5 μg/g creatinine, which is still in application in most countries, was set on the basis of this CdU threshold value after the application of a safety margin of two to account for inter-individual variations in the renal toxicity of Cd.

Since then, a number of studies have further explored the dose–effect/response relationships for

Cd-induced renal dysfunction in industrial workers and in the general population.[1 7–12] Although utilising the same biomarkers of exposure and renal effects (mostly CdU and β_2-mU), some studies have derived increasingly lower CdU thresholds.[13–16] (for review see also Anon[17]). Recent studies among children and the elderly with low-level mixed exposure to elements including Cd even suggest that Cd can induce LMW proteinuria at CdU<1 μg/g creatinine, that is at the exposure levels currently prevailing in industrialised countries.[14 18]

Differences in sensitivity between workers and some groups of the general population, especially the elderly and children, have been proposed to explain such a wide variation in CdU thresholds. An alternate explanation, which has received little consideration even in the most recent risk assessments,[17] might be insufficient adjustment for potential confounders such as smoking, ageing and the co-excretion of Cd with urinary proteins.[1 19] In most studies addressing the renal toxicity of Cd, smoking has been regarded as merely an additional source of Cd exposure having no other influence except to increase the Cd body burden. This long-standing view is now challenged by studies showing that tobacco smoke is detrimental to renal function, even in subjects without hypertension or abnormal glucose metabolism.[20–23] Chronic smoking, even when moderate, is associated with a marked increase in the urinary excretion of albumin, which reflects glomerular damage linked most likely to the cardiovascular effects of tobacco smoking.[22 23] This renal damage, although distinct from the tubular dysfunction caused by Cd, might substantially distort the dose–response relationships between LMW proteins and Cd in urine. Confounding might arise because of the co-excretion of Cd with albumin, the main Cd-binding protein in plasma,[24] or else with LMW proteins since tubulo-interstitial involvement is common in glomerulopathies.[25 26] In population-based studies of the elderly, relationships between LMW proteins and Cd in urine might be further distorted by changes in Cd metabolism and renal function occurring with advanced age. It is well established that the renal burden of Cd and thus urinary excretion of the metal, decrease after the age of 60–70 years,[1] which consequently displaces the dose–effect/response relationships to the left with resulting underestimation of the CdU threshold value. Ageing is also accompanied by abnormalities in the urinary excretion of albumin and LMW proteins,[27] which may generate secondary associations because of the co-excretion of Cd with albumin and/or LMW proteins. Confounding might also arise from the residual influence of diuresis, since concentrations of Cd and LMW proteins expressed per gram of creatinine can still correlate with urinary creatinine.[19] Last, the proteolytic degradation of β_2-m in acidic urine, which starts in the bladder,[28] is an additional source of confounding that increases the inter-individual variability of this biomarker and probably explains why β_2-mU is higher in Asian than in Caucasian populations.[17]

The objective of the present study was to carry out a refined analysis of the dose–effect/response relationships between LMW proteins and Cd in the urine of Cd-exposed workers to determine to what extent renal dysfunction is likely to occur at CdU levels below the current occupational exposure limit of 5 μg/g creatinine. We focused our study on active workers as Cd excretion is reduced after exposure ceases.[29] Different models were tested to ensure that associations were not distorted by smoking or diuresis. We also consolidated our analysis by screening renal dysfunction on the basis of β_2-mU and RBPU, a much more stable biomarker of tubular dysfunction.[30]

MATERIALS AND METHODS

Study population

The studied population comprised 599 active workers employed in four nickel-cadmium battery plants located in France, Sweden and the USA. Biological data and information on the duration of employment and smoking history were obtained in the framework of the workers' health surveillance programme that was implemented in each factory in compliance with national regulations. These data were supplied anonymously by the factory health care units. The studied database included all workers examined during 2008–2009 and for whom one complete set of values for urinary Cd and RBP or β_2-m was available. We excluded one outlier worker who had a normal CdU value (1.9 μg/g creatinine) but very high concentrations of RBP and β_2-m in urine (>5000 μg/g creatinine). A total of 135 workers had a CdU value above the occupational exposure limit (5 μg/g creatinine) including 19 workers who had been removed from Cd exposure. As these workers had CdU levels similar to those of their colleagues still exposed to Cd (n=116) (median (interquartile range, IQR), 8.1 (6.9–9.7) vs 7.5 (6.0–10.3), p=0.72), we decided to retain them in the final cohort. When several sets of data were available, we selected the most recent with a urinary creatinine value close to 1.

Biomarkers

Analyses were performed on untimed urine specimens sent immediately to our laboratory and kept frozen until protein analyses. We measured the concentrations of β_2-m and RBP by latex immunoassay with detection and quantification limits of 0.5 and 2.5 μg/l, respectively, based on a fivefold dilution of urine.[31 32] Because of practical constraints, pH could not be measured in urine samples immediately after collection or in the laboratory since most samples were buffered by adding 10% (vol/vol) of a 1 mol/l phosphate buffer pH 7.4. Cd was measured in urine by means of inductively coupled argon plasma mass spectrometry with an Agilent 7500 instrument (Agilent Technologies. Santa Clara, California, USA). Briefly, urine specimens (500 μl) were diluted quantitatively (1+9) with a HNO_3 1%, HCl 0.5% solution containing Sc, Ge, Rh and Ir as internal standards. The detection and quantification limits were 0.02 and 0.05 μg/l, respectively. Using this method, the laboratory has obtained successful results in external quality assessment schemes with a certificate (2008–2009) awarded by the Institute for Occupational, Environmental and Social Medicine of the University of Erlangen, Germany (G-EQUAS programme) and 100% performance (2008–2009) achieved in the PCI and QMEQUAS programmes organised by the Institut National de Santé Publique, Québec. Creatinine was determined by a modified Jaffé reaction using a Beckman Synchron LX 20 analyser (Beckman Coulter, Krefeld, Germany). This method allows to minimize interference by protein, bilirubin and glucose.[33 34]

Statistics

Concentrations of creatinine, Cd and LMW proteins in urine were presented as medians with IQRs and normalised by log-transformation. Age, duration of exposure and number of pack-years were presented as means with SDs. Associations between CdU, age and biomarkers of renal function were assessed by Pearson's correlation analysis. To analyse the associations between these variables and smoking dose (pack-years), we used the non-parametric Spearman's rank correlation coefficient. Factors influencing RBPU and β_2-mU were identified by stepwise regression analysis by testing as potential predictors

age, gender, urinary creatinine, CdU and smoking defined either as never/ever smoking or as categories of pack-years (never smoking: 0, low: >1–10, medium: >10–20, and high: >20) introduced into the models as dummy variables. We also assessed the interactions of CdU and urinary creatinine with gender and smoking, as well as the interaction between gender and smoking. When using the number of pack-years to assess cumulative exposure to tobacco smoke, current smoking was added to the list of predictors. Potential predictors were entered at $p<0.25$ and retained in the final model at $p<0.1$. When age and gender did not emerge as predictors at $p<0.1$, they were nevertheless forced into the models on the basis of a priori knowledge. We performed these analyses by testing three different models to adjust for the residual effect of diuresis. In a first model, we expressed LMW proteins and Cd per gram of creatinine and excluded urine samples with creatinine values <0.3 and >3 g/l. In the second model, LMW proteins and CdU were also expressed per gram of creatinine, but we considered urinary creatinine as an independent variable. In this model, CdU was tested after a preliminary adjustment for urinary creatinine on the basis of the simple regression coefficient in order to avoid any collinearity and confounding by a residual influence of diuresis. In the third model, we expressed the concentrations of LMW proteins and Cd per litre, considering urinary creatinine as a separate independent variable as suggested by Barr *et al*.[35] Since concentrations of β_2-mU below 20 μg/g creatinine usually reflect proteolytic degradation due to pH<5.6,[28] we performed these statistical analyses on all values of β_2-mU and after the exclusion of values <20 μg/g creatinine. In all these regression models, collinearity was excluded by calculating the variance inflation factor.

To estimate the CdU threshold for renal dysfunction, we stratified workers into seven categories of increasing CdU values (μg/g creatinine): ≤1, >1–2, >2–3, >3–4, >4–6, >6–10 and >10. These categories were based on an increment of one unit. For the most exposed workers, increments of two and four were used to obtain sufficient number of subjects per category. After adjustment for their respective covariates, RBPU and β_2-mU were compared between these categories by one-way ANOVA followed by the Tukey–Kramer post hoc test. Risks of increased urinary RBP and β_2-m were then assessed by logistic regression analysis using as reference level the prevalence of values exceeding the 95th percentile of urinary concentrations (adjusted for covariates) among workers with CdU<1 μg/g creatinine. We used a backward approach by including as potential predictors the same independent variables as in multiple regression analyses and removing the least significant predictor until the model contained only variables with $p<0.20$. Threshold values of CdU were then estimated by calculating the benchmark dose (BMD5) and the benchmark dose lower CI limit (BMDL5) for a 5% excess in the background prevalence of abnormal RBPU and β_2-mU. We used the Hill model as provided by the software (BMDS v 2.1.1) developed by the US Environmental Protection Agency. The BMD5 corresponds to the lowest observed adverse effect level (LOAEL) or to the population-based critical level used in previous studies (a 10% response for a background prevalence of 5%), while the BMDL5 can be assimilated to the no observed adverse effect level (NOAEL). Never/ever smokers across CdU categories were compared using the χ^2 test. The level of statistical significance was set at $p<0.05$. Statistical analyses were performed by using SAS v 9.1.3.

RESULTS

Table 1 gives the characteristics and biomarker levels of workers from the four nickel-cadmium battery plants. The population included 24.7% women and 44.6% ever smokers. Cumulative exposure to Cd, as reflected by CdU, was higher in the French plants, in particular plant 1 where median CdU was about five times higher than the background level in the general population. Mean urinary excretion of RBP paralleled CdU, also being highest in plant 1 in France. Urinary excretion of Cd (μg/g creatinine) was higher in women than in men (median (IQR) 3.40 (1.16–7.46) vs 1.61 (0.62–3.57), $p<0.001$) and in ever smokers compared to never smokers (median (IQR) 2.09 (0.76–4.55) vs 1.67 (0.74–3.91), $p=0.048$). Urinary RBP (μg/g creatinine) was higher in ever than in never smokers (median

Table 1 Characteristics of nickel-cadmium battery workers and biological parameters

	France (plant 1)	France (plant 2)	Sweden	USA	Total
N	251	221	111	16	599
Women, n (%)	58 (23.1)	49 (22.2)	35 (31.5)	6 (37.5)	148 (24.7)
Age, mean (SD), years	47.8 (6.6)	45.5 (11.3)	40.1 (13.0)	44.9 (7.2)	45.4 (10.3)
Years of exposure, mean (SD)	22.4 (8.1)	18.3 (13.2)	13.7 (10.2)	13.4 (9.7)	18.8 (11.3)
Never smoker, n (%)	154 (61.4)	107 (48.4)	59 (53.2)	12 (75.0)	332 (55.4)
Ex-smokers					
n (%)	27 (10.8)	31 (14.0)	24 (21.6)	4 (25.0)	86 (14.3)
Pack-years, mean (SD)	8.3 (7.7)	15.5 (13.7)	11.0 (8.4)	14.9 (8.3)	11.9 (10.7)
Current smokers					
n (%)	70 (27.9)	83 (37.6)	28 (25.2)	0 (0)	181 (30.2)
Pack-years, mean (SD)	15.1 (10.2)	16.2 (8.2)	9.3 (9.8)	0 (0)	14.7 (9.5)
Urinary creatinine, median (IQR), g/l	1.37 (0.93–1.80)	1.13 (0.69–1.70)	1.47 (1.08–1.90)	1.53 (1.07–2.15)	1.33 (0.86–1.80)
Urinary Cd, median (IQR)					
μg/l	4.72 (2.23–7.84)	1.27 (0.54–2.75)	1.08 (0.49–2.74)	1.48 (0.82–2.73)	2.07 (0.86–5.42)
μg/g creatinine	3.40 (1.74–6.40)	1.24 (0.51–2.81)	0.81 (0.31–1.92)	0.98 (0.73–1.31)	1.82 (0.75–4.11)
Urinary RBP, median (IQR)					
μg/l	168 (108–295)	136 (82.0–219)	145 (85.6–215)	117 (64.5–206)	152 (88.5–252)
μg/g creatinine	129 (91.5–194)	119 (80.0–180)	100 (69.0–140)	83.8 (57.0–102)	117 (80.4–176)
Urinary, β_2-m*, median (IQR)					
μg/l	80.0 (36.0–153)	72.7 (48.2–119)	81.5 (52.0–131)	88.0 (26.0–135)	79.0 (43.0–132)
μg/g creatinine	69.0 (33.9–132)	75.5 (31.7–137)	59.2 (39.5–89.8)	62.5 (9.90–90.1)	66.1 (33.5–122)

*n=228, 152, 98, 14 for total of 492.
β_2-m, concentration of β_2-microglobulin; Cd, concentration of cadmium; IQR, interquartile range; RBP, concentration of retinol-binding protein.

(IQR) 129 (88.6–201) vs 109 (77.6–161), p=0.03) while urinary β_2-m (μg/g creatinine) was higher in women than in men (median (IQR) 89.2 (48.4–146) vs 58.6 (31.2–113), p=0.006). Smoking history expressed in pack-years was also different between women and men (mean (SD) 4.8 (7.5) vs 6.6 (10.1), p=0.001). It can be seen from table 1 that in relative terms the inter-individual variability of β_2-mU is greater than that of RBPU, most probably reflecting the differences in stability between the two urinary proteins.

The univariate associations between creatinine, Cd, RBP and β_2-m in urine as well as those with age and pack-years are shown in table 2. RBPU and β_2-mU (all values or only values >20 μg/g creatinine), expressed per litre or per gram of creatinine, were significantly correlated with both CdU and number of pack-years. Of note, the urinary excretion of Cd, RBP and β_2-m expressed per gram of creatinine showed a highly significant negative correlation with urinary creatinine, a residual association that persisted after exclusion of extreme values of urinary creatinine (<0.3 or >3.0 g/l, all p<0.001; results not shown).

Because of this residual influence of urinary creatinine on the concentrations of Cd and LMW proteins expressed per gram of creatinine, we studied factors influencing the excretion of RBP and β_2-m by testing three models of correction for urinary creatinine (tables 3 and 4). In the first two models, Cd and proteins were expressed per gram of creatinine. To account for the residual influence of diuresis in these two models, we either eliminated samples with extreme values of creatinine (model 1) or we considered urinary creatinine as a predictor while further adjusting CdU for urinary creatinine in order to avoid the collinearity among predictors shown in table 2 (model 2). In a third model as proposed by Barr *et al*,[35] Cd and proteins were expressed per litre and urinary creatinine was added to the list of potential predictors. As shown in table 3, whatever the model selected to adjust for urinary creatinine and for smoking (pack-years categories or ever vs never smokers), RBPU showed consistent positive associations with CdU, age, gender (male sex) and ever smoking or categories of pack-years. For β_2-mU, in contrast, determinants varied depending on the model adopted (table 4). The urinary excretion of β_2-m increased significantly with both CdU and smoking only in the models based on pack-years categories and on samples with β_2-mU >20 μg/g creatinine. CdU was the only significant predictor emerging in the other models. The same pattern of associations between LMW proteins and Cd in urine was found when restricting the analysis to male workers (results not shown).

Since correction for urinary creatinine is the most commonly used method to adjust for variation in diuresis in biomonitoring, we pursued our analysis with the concentrations of LMW proteins and Cd in urine expressed per gram of creatinine and adjusted according to model 2 based on pack-years categories. For β_2-mU, we selected the same model 2 but with β_2-mU values >20 μg/g creatinine. We first examined whether the associations between LMW proteins and Cd in urine differed between ever and never smokers and could be detected at low CdU values or only above a certain threshold. We found that RBPU and β_2-mU correlated significantly with CdU in workers with CdU<2 μg/g creatinine, and thus in subjects with no or minimal occupational exposure to Cd. However, as shown in online figures 1A and B, these associations were largely driven by smoking as they were absent in never smokers (RBPU, p=0.26; β_2-mU, p=0.21), while they were strengthened among ever smokers (RBPU and β_2-mU, p=0.02).

Dose–effect/response relationships were then assessed by stratifying workers in seven categories of increasing CdU (μg/g creatinine) using as referents subjects with CdU<1. The 95th percentile adopted as the upper limit of normal showed little variation whether calculated on all subjects or on never and ever smokers separately (RBPU, 256.2, 256.4, 255.5 μg/g creatinine; β_2-mU, 276.4, 266.5, 252.5 μg/g creatinine). Although the proportions of never/ever smokers did not vary across these CdU categories both for RBPU (p=0.67) and β_2-mU (p=0.89), the dose–effect relationships were strongly influenced by smoking as depicted in online figure 2. Among never smokers the urinary excretion of both RBP and β_2-m showed a sharp and very significant increase at CdU>10. When considering all workers, RBPU adjusted for covariates including pack-years increased significantly from CdU>6–10. In ever smokers, RBPU rose significantly again from CdU>10, but no significant trend was seen for β_2-mU.

This residual influence of smoking despite adjustment for pack-years emerges more noticeably when analysing dose–response relationships between CdU categories (μg/g creatinine) and the prevalences of increased values of RBPU and β_2-mU. When considering all workers regardless of their smoking status, the odds for abnormal values of RBPU were significantly increased in subjects with CdU>6–10 (OR 3.9, 95% CI 1.6 to 9.6) as well as in those with CdU>10 (OR 13.3, 95% CI 5.2 to 34.2). The same pattern of increase was observed with the odds of abnormal β_2-mU (CdU>6–10: OR 2.8, 95% CI 0.9 to 8.6; CdU>10: OR 9.4, 95% CI 3.2 to 27.9). The

Table 2 Correlations between studied parameters

	Age	Pack-years†	Log CdU (μg/l)	Log CdU (μg/g creatinine)	Log CrU (μg/l)	Log RBPU (μg/l)	Log RBPU (μg/g creatinine)	Log β_2-mU (μg/l)	Log β_2-mU (μg/l)>20
Age	–								
Pack-years†	0.10*								
Log CdU, μg/l	0.39***	0.15***							
Log CdU, μg/g creatinine	0.52***	0.18***	0.88***						
Log CrU, μg/l	−0.26***	0.06	0.25***	−0.24***					
Log RBPU, μg/l	0.01	0.18***	0.37***	0.07	0.60***				
Log RBPU, μg/g creatinine	0.24***	0.22***	0.23***	0.30***	−0.14***	0.71***			
Log β_2-mU, μg/l	0.02	0.15**	0.10*	0.07	0.05	0.36***	0.42***		
Log β_2-mU, μg/g creatinine	0.14**	0.16***	−0.03	0.18***	−0.45***	0.01	0.43***	0.87***	
Log β_2-mU>20 μg/l	0.03	0.18***	0.28***	0.15**	0.28***	0.59***	0.51***	–	
Log β_2-mU>20 μg/g creatinine	0.21***	0.19***	0.10*	0.30***	−0.42***	0.11*	0.55***	–	0.76***

r Values are Pearson's correlation coefficients.
*p<0.05; **p<0.01; ***p<0.001.
†Indicates Spearman's rank correlation coefficients with number of pack-years.
β_2-mU, concentration of β_2-microglobulin in urine; CdU, concentration of cadmium in urine; CrU, concentration of creatinine in urine; RBPU, concentration of retinol-binding protein in urine.

Table 3 Factors influencing urinary excretion of retinol-binding protein in nickel-cadmium battery workers

	Categories of pack-years		Never/ever smoking	
	Independent variables	**Coefficient (95% CI)**	**Independent variables**	**Coefficient (95% CI)**
Model 1				
Log RBPU	Log CdU (μg/g CrU)	0.142 (0.090 to 0.194)	Log CdU (μg/g CrU)	0.143 (0.091 to 0.195)
(μg/g CrU)	Age (years)	0.003 (<0.001 to 0.006)	Age (years)	0.004 (0.001 to 0.007)
	Gender	0.064 (0.005 to 0.122)	Gender	0.074 (0.016 to 0.132)
	Category of pack-years (medium)	0.075 (0.009 to 0.142)	Ever smoking	0.059 (0.010 to 0.107)
	Category of pack-years (high)	0.096 (0.006 to 0.186)		
Model 2				
Log RBPU	Log CdU (μg/g CrU) CrU adjusted	0.137 (0.085 to 0.188)	Log CdU (μg/g CrU) CrU adjusted	0.138 (0.087 to 0.189)
(μg/g CrU)	Log CrU (g/l)	−0.180 (−0.278 to −0.082)	Log CrU (g/l)	−0.186 (−0.284 to −0.089)
	Age (years)	0.003 (−0.0001 to 0.005)	Age (years)	0.003 (0.001 to 0.006)
	Gender	0.097 (0.038 to 0.156)	Gender	0.107 (0.049 to 0.166)
	Category of pack-years (medium)	0.058 (−0.007 to 0.122)	Ever smoking	0.054 (0.007 to 0.101)
	Category of pack-years (high)	0.090 (0.004 to 0.177)		
Model 3				
Log RBPU	Log CdU (μg/l)	0.137 (0.085 to 0.188)	Log CdU (μg/l)	0.138 (0.087 to 0.189)
(μg/l)	Log CrU (g/l)	0.750 (0.644 to 0.856)	Log CrU (g/l)	0.743 (0.638 to 0.849)
	Age (years)	0.003 (0.0001 to 0.005)	Age (years)	0.003 (0.001 to 0.006)
	Gender	0.097 (0.038 to 0.156)	Gender	0.107 (0.049 to 0.166)
	Category of pack-years (medium)	0.058 (−0.007 to 0.122)	Ever smoking	0.054 (0.007 to 0.101)
	Category of pack-years (high)	0.090 (0.004 to 0.177)		

Model 1, N=568; models 2 and 3, N=599.
Model 1: RBPU and CdU expressed per gram creatinine and exclusion of urine samples with creatinine <0.3 and >3 g/l.
Model 2: RBPU and CdU expressed per gram creatinine but with creatinine tested as an independent variable and CdU further adjusted for creatinine on the basis of the simple regression coefficient.
Model 3: RBPU and CdU expressed per litre with creatinine tested as an independent variable.
CdU, concentration of cadmium in urine; CrU, concentration of creatinine in urine; RBPU, concentration of retinol-binding protein in urine.

benchmark dose (BMD5) and the benchmark dose lower limit (BMDL5) for a 5% excess in the background prevalence of abnormal RBPU and β_2-mU were estimated at 5.1/3.0 and 9.6/5.9. When excluding ever smokers from the analysis, the odds for abnormal RBPU and β_2-mU were significantly increased only among workers with CdU>10 (RBPU: OR 21.8, 95% CI 6.4 to 74.4; β_2-mU: OR 15.1, 95% CI 3.6 to 63.1). The BMD5/BMDL5 values for abnormal RBPU and β_2-mU in never smokers were estimated at 12.6/6.6 and 12.2/5.5. Among ever smokers, RBPU and β_2-mU showed a significant increase from CdU>6–10 (RBPU: OR 5.8, 95% CI 1.6 to 20.3; β_2-mU: OR 5.6, 95% CI 1.3 to 24.6) and CdU>10 (RBPU: OR 5.5, 95% CI 1.23 to 25.0; β_2-mU: OR 5.0, 95% CI 0.9 to 28.5). The BMD5/BMDL5 values for abnormal RBPU and β_2-mU in ever smokers were assessed at 6.3/4.9 and 4.3/3.5, respectively.

If as suggested by our findings, BMD values are artificially decreased by the renal effects of smoking unrelated to Cd, then in subjects with CdU below the BMDL5 estimated in never

Table 4 Factors influencing urinary excretion of β_2-microglobulin in nickel-cadmium battery workers

	Categories of pack-years		Never/ever smoking	
	Independent variables	**Coefficient (95% CI)**	**Independent variables**	**Coefficient (95% CI)**
Model 1				
Log β2-mU (μg/g CrU)	Log CdU (μg/g CrU)	0.120 (0.022 to 0.218)	Log CdU (μg/g CrU)	0.120 (0.022 to 0.219)
			Gender	−0.101 (−0.217 to 0.014)
Model 1 with β2-mU>20 μg/g CrU				
Log β2-mU (μg/g CrU)	Log CdU (μg/g CrU)	0.166 (0.090 to 0.243)	Log CdU (μg/g CrU)	0.177 (0.100 to 0.253)
	Category of pack-years (high)	0.166 (0.025 to 0.308)		
Model 2				
Log β2-mU (μg/g CrU)	Log CdU (μg/g CrU) CrU adjusted	0.083 (−0.006 to 0.173)	Log CdU (μg/g CrU) CrU adjusted	0.078 (−0.005 to 0.174)
	Log CrU (g/l)	−0.937 (−1.113 to −0.760)	Log CrU (g/l)	−0.907 (−1.02 to −0.429)
Model 2 with β2-mU>20 μg/g CrU				
Log β2-mU (μg/g CrU)	Log CdU (μg/g CrU) CrU adjusted	0.150 (0.078 to 0.222)	Log CdU (μg/g CrU) CrU adjusted	0.153 (0.091 to 0.215)
	Log CrU (g/l)	−0.638 (−0.777 to −0.498)	Log CrU (g/l)	−0.614 (−0.706 to −0.249)
	Category of pack-years (high)	0.144 (0.017 to 0.271)		
Model 3				
Log β2-mU (μg/l)	Log CdU (μg/l)	0.083 (−0.001 to 0.177)	Log CdU (μg/g CrU)	0.088 (−0.001 to 0.177)
Model 3 with β2-mU>20 μg/g CrU				
Log β2-mU (μg/l)	Log CdU (μg/l)	0.150 (0.078 to 0.222)	Log CdU (μg/l)	0.153 (0.091 to 0.215)
	Log CrU (g/l)	0.286 (0.135 to 0.436)	Log CrU (g/l)	0.308 (0.179 to 0.437)
	Category of pack-years (high)	0.144 (0.017 to 0.271)		

The models are the same as described in table 3.
For each model, age and gender were forced in the regression.
Model 1, N=468; model 1 with β_2-mU>20 μg/g CrU, N=393; models 2 and 3, N=492; models 2 and 3 with β_2-mU>20 μg/g CrU, N=416.
β_2-mU, concentration of β_2-microglobulin in urine; CdU, concentration of cadmium in urine; CrU, concentration of creatinine in urine.

smokers, the excretion of RBPU and β_2-mU adjusted for covariates including CdU should increase with categories of pack-years. We checked the validity of this assumption by performing the same analyses as above but only among subjects with CdU<BMDL5 (6.6 μg/g creatinine). Table 5 shows that in men the risk of increased RBPU and the mean concentration of β_2-m rose dose dependently across categories of pack-years. Of note, the workers who had smoked moderately (10–20 pack-years) were three times more likely to have increased excretion of RBPU (OR 3.11, 95% CI 1.28 to 7.55) while being the same age and having the same CdU as never smokers. Such associations were not found in women (all p>0.17; data not shown).

DISCUSSION

The primary goal of this study was to re-assess the threshold value of urinary Cd for renal dysfunction by taking into account confounders that might explain the discordant results between studies involving industrial workers and those based on the general population. Potential confounders tested in our study included age, gender, smoking and the residual influence of diuresis after correction for urinary creatinine. We also took into account the bias that might result from the instability of urinary β_2-m by using RBPU, a much more stable biomarker, as well as by excluding urine samples with abnormally low values of β_2-mU. Our results show that chronic smoking causes a shift in the dose–effect/response relationship, which leads to a significant underestimation of the CdU threshold value. The BMD5 values of CdU for increased β_2-mU and RBPU derived in never smokers (~12 μg/g creatinine) were two or three times higher than those calculated among ever smokers. Such differences persisted in the whole population despite adjustment of urinary LMW proteins for smoking status or pack-years smoking history, which means that a reliable evaluation of the BMD can be performed only by excluding ever smokers from the analysis.

Confounding of the associations between LMW proteins and Cd in urine appears to stem primarily from the renal damage associated with chronic smoking.[20–23] Cumulative exposure to tobacco smoke evaluated on the basis of pack-years was positively associated with urinary excretion of both RBP and β_2-m. Interestingly, this tubular dysfunction associated with smoking emerged in men but not in women, a phenomenon also described by Briganti *et al*[22] and Orth *et al*.[36]

The exact mechanism by which tobacco smoking causes renal damage and distorts the relationships between LMW proteins and Cd in urine is unknown. Our findings suggest that the LMW proteinuria associated with chronic smoking is unlikely to be caused by Cd alone. In multivariate analyses, the associations of RBPU and β_2-mU with pack-years history emerge concurrently with the association of these proteins with CdU, which however integrates exposure to Cd from all sources, including tobacco smoke. The fact that cumulative smoking is associated with LMW proteinuria only in men also argues against Cd being the sole causative agent since there is no evidence that men are more sensitive to Cd than women.[1] As to the shift of the dose–response relationships to lower CdU values observed in ever smokers, we think that it might result from two distinct, although not exclusive, mechanisms. One mechanism might be an effect modification similar to that proposed for the Cd–diabetes interaction reported previously.[1 8] According to this mechanism, the renal impairment associated with smoking would make the kidney more sensitive to Cd toxicity, thereby decreasing the CdU threshold. The second mechanism might be a distortion of the dose–response curve by secondary associations linked to the toxicokinetics of Cd. For instance, an association between urinary Cd and renal dysfunction in smokers might develop secondarily to the accumulation and progressive rise in urinary Cd that occurs during chronic smoking. Another possibility directly involving the glomerular damage[22 23] associated with chronic smoking would be enhanced co-excretion of Cd with albumin, the main Cd-binding protein in plasma.[24] A third possibility would be increased co-excretion of Cd with LMW proteins, in particular with metallothionein, which has been shown to follow the same glomerular filtration–tubular reabsorption pathway as other LMW proteins.[37] The increased excretion of metallothionein following tubular damage is a well-known phenomenon which has been demonstrated in experimental animals.[38] The decreased renal uptake of proteins in subjects with glomerular proteinuria might be the consequence of tubular damage, but it might also simply result from competitive inhibition of tubular reabsorption of LMW proteins by the high filtered load of albumin and other high molecular weight proteins.[39] In this third mechanism, which

Table 5 Risks of low-molecular-weight proteinuria in male workers with CdU<BMDL5 (6.6 μg/g creatinine) according to cumulative smoking

		Categories of pack-years			
	Never smokers	<10	10–20	>20	p Value
N*	232	61	69	39	
Age, mean (SD), years	44.0 (10.0)	40.1 (8.8)	42.0 (10.7)	52.5 (6.7)§	<0.001
Cd in urine					
Median (IQR), μg/g creatinine adjusted for CrU	1.40 (0.57–2.90)	1.23 (0.55–2.75)	1.15 (0.52–3.08)	2.63 (1.52–3.76)§	0.004
RBP in urine adjusted for age, CrU and CdU†					
Median (IQR), μg/g creatinine	113 (78–160)	135 (99.8–178)	143 (96.7–200)§	141 (86–205)	0.03
N>285 μg/g creatinine (%)	12 (5.2)	3 (4.9)	10 (14.5)	4 (10.3)	0.03
OR (95% CI)	1.00 (1.0 to 1.0)	0.95 (0.26 to 3.47)	3.11 (1.28 to 7.55)	2.10 (0.64 to 6.86)	
β_2-Microglobulin in urine adjusted for CrU and CdU†, ‡					
Median (IQR), μg/g creatinine	78.7 (54.5–120)	77.0 (55.2–144)	92.7 (62.3–149)	121 (80.7–231)§	0.02
N>295 μg/g creatinine (%)	8 (5.1)	3 (7.0)	3 (5.9)	2 (7.7)	0.59
OR (95% CI)	1.0 (1.0 to 1.0)	1.41 (0.36 to 5.55)	1.17 (0.30 to 4.60)	3.41 (0.31 to 7.81)	

p Values indicate the level of statistical significance in the χ^2 test for trend (prevalences) or in one-way ANOVA.
*For β_2-mU, n=158, 43, 51 and 26.
†Adjustment based on the multiple regression analysis of data in workers with CdU<6.6 μg/g creatinine.
‡β_2-mU>20 μg/g CrU.
§Denotes statistical significance (p<0.05) by comparison with never smokers (Tukey-Kramer post hoc test).
β_2-mU, concentration of β_2-microglobulin in urine; BMDL5, benchmark dose lower limit for a 5% excess in the background prevalence of abnormal RBPU and β_2-mU; CdU, urinary cadmium; RBPU, retinol-binding protein.

might apply to situations other than smoking, CdU would thus be a reflection more of the functional integrity of the proximal tubule than of the Cd body burden. We are currently exploring these different mechanisms by assessing the impact of high albumin excretion on the relationships between LMW proteins and Cd in urine from Cd-exposed workers and from populations with low environmental exposure to Cd.[40]

Smoking, although having a very significant influence, is not the only factor that may bias risk assessment of Cd. As shown in our study, excretion of both Cd and LMW proteins is still influenced by diuresis after correction for urinary creatinine. In addition to interacting positively with smoking to increase the excretion of LMW proteins in urine, gender appears to have an independent influence on the excretion of RBP, which was slightly higher in men than in women. We found, however, no evidence of an interaction between gender and CdU regarding the risk of LMW proteinuria, suggesting that men and women present the same sensitivity to the nephrotoxic effects of Cd. Other potential sources of confounding, which were not really relevant to the present study, include ageing and the decrease in Cd body burden after removal from exposure.

The strength of our study lies mainly in the fact that it has been performed under conditions which do not require the use of uncertainty or adjustment factors for setting occupational exposure limits in contrast to risk assessments based on aggregate or extrapolated data.[17] Since our study involved only active workers, adjustment for the loss of Cd occurring after retirement was not necessary.[29] The studied cohort was also sufficiently large to allow estimation of threshold values for Cd-induced LMW proteinuria using data from never smokers only, which seems to be the most reliable way to derive associations unconfounded by smoking. The use of RBPU and β_2-mU which we measured by means of very sensitive assays, represents another strength in comparison with those studies which measured only one LMW protein, most frequently β_2-mU, a protein unstable in acid urine or else protein HC, a more stable but less specific indicator of tubular dysfunction.

Our study, however, has some limitations. Since data were collected in the framework of medical surveillance in the workplace, we did not have access to workers' complete medical files, so some information on disease likely to affect renal function or to modify the renal response to Cd could not be retrieved (eg, hypertension, diabetes, urinary tract infection, etc). Nor did we collect data on alcohol consumption, physical activity and possible exposure to other nephrotoxicants at home. Concentrations of albumin or total protein in urine were not available for all participants, which would have permitted direct adjustment for the glomerular damage caused by smoking or other chronic diseases unrelated to Cd.

In conclusion, our study provides evidence that BMD values derived from populations including smokers do not represent the true threshold for the renal dysfunction induced by Cd even when data are adjusted for smoking status or cumulative exposure to tobacco smoke. Because of the renal effects of smoking unrelated to Cd, dose–effect/response relationships between LMW proteins and Cd in urine are shifted to lower CdU values. When dose–effect/response relationships are not distorted by the renal toxicity of tobacco smoke and adjusted for age, sex and the residual influence of diuresis, the BMDL5 values of CdU for LMW proteinuria induced by occupational exposure to Cd can be reliably estimated between 5.5 and 6.6 μg/g creatinine. The corresponding BMD5 values are estimated around 12 μg/g creatinine, which is in agreement with the critical concentration of 10 μg/g creatinine derived in earlier studies.

Funding Alfred Bernard is Research Director of the National Fund for Scientific Research in Belgium. The study was supported by the Reach Cadmium Consortium and the European Union (FP6 Phime project, coordinator Staffan Skerfing). The funders of the study had no role in the data analysis, data interpretation and writing of the report.

Competing interests None.

Provenance and peer review Not commissioned; externally peer reviewed.

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