

## The influence of cadmium exposure on excretion of pyridinoline and deoxypyridinoline in urine

### Wpływ narażenia na kadm na wydalanie pirydynoliny i dezoksypirydynoliny z moczem

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(a) idea

(b) determination of metals

(c) determination of crosslinks

(d) data analysis

(e) text and references

#### ABSTRACT

**Background:** Osteoporosis is a growing health concern across the world. Some epidemiological data suggest that cadmium increases risk for development of osteoporosis and lead to higher rate of fracture incidents even on low environmental exposure level. **Material and methods:** Cadmium in urine and bone resorption markers – total fraction of the urinary pyridinoline (Pyr) and deoxypyridinoline (DPyr) – were determined in 36 patients, who were examined for toxic effects of cadmium exposure. Additionally calcium in urine was determined. Associations between cadmium exposure and factors related to bone metabolism were estimated and Pyr and DPyr excretion were compared in three groups categorized across cadmium concentrations. **Results:** In the investigated group there were significant positive correlations between cadmium levels in urine and Pyr and DPyr excretion. None of the other variables correlated significantly with examined bone resorption markers excluding calcium excretion in urine. Excretion of Pyr and DPyr differed significantly between group with the lowest cadmium concentration (< 1.2 µg/g creatinine) and group with the highest cadmium concentration (> 1.9 µg/g creatinine), where median values of Pyr and DPyr increased by 49.8% and 37.5%, respectively. **Conclusion:** The results suggest that cadmium increases bone resorption processes and induce osteotoxic effects in environmental exposure level.

**Key words:** cadmium, osteoporosis, pyridinoline, deoxy-pyridinoline

#### STRESZCZENIE

**Wstęp:** Osteoporoza jest rosnącym problemem na świecie. Niektóre badania epidemiologiczne sugerują, że kadm zwiększa ryzyko rozwoju tej choroby i prowadzi do zwiększenia ryzyka złamań kości nawet przy niskim narażeniu środowiskowym. **Materiał i metody:** Kadm w moczu oraz biomarkery resorpcji kostnej – całkowite frakcje pirydynoliny (Pyr) i dezoksypirydynoliny (DPyr) w moczu – były oznaczane u 36 pacjentów badanych pod kątem toksycznego działania kadmu. Dodatkowo oznaczano zawartość wapnia w moczu. Oszacowano zależności pomiędzy narażeniem na kadm a czynnikami powiązаныmi z metabolizmem kości oraz porównano stężenia Pyr oraz DPyr pomiędzy grupami skategoryzowanymi względem stężenia kadmu w moczu. **Wyniki:** W badanej grupie wykazano statystycznie istotną pozytywną korelację pomiędzy poziomem kadmu w moczu, a ilością wydalanej Pyr i DPyr z moczem. Żadna z innych badanych zmiennych nie korelowała znacząco z markerami resorpcji kości oprócz wydalania wapnia z moczem. Zróżnicowanie wydalania Pyr i DPyr było statystycznie istotne pomiędzy grupami o najniższym (< 1.2 µg/g kreatyniny) i najwyższym stężeniu kadmu w moczu (> 1.9 µg/g kreatyniny), w której mediany wartości stężeń Pyr i DPyr wzrosły odpowiednio o 49,8% i 37,5%. **Wnioski:** Wyniki badań sugerują, że kadm wzmacnia procesy resorpcji kości i działa osteotoksycznie również w narażeniu środowiskowym.

**Słowa kluczowe:** kadm, osteoporoza, pirydynolina, dezoksypirydynolina

## INTRODUCTION

Cadmium is a widespread and persistent contaminant, occurring in the environment from natural processes and human activities. Current and past emission from non-metal industry, emission from waste incineration and fossil fuel combustion, as also application of phosphorus fertilizers contaminated by cadmium and fertilizers originated from sewage sludge are recognized as the main sources of cadmium pollution [1]. Human exposure to cadmium is mainly via the food, especially by the consumption of shellfish, offal products, cereals and vegetables [2]. Direct inhalation of cigarette smoke causes additional exposure to high amount of cadmium through the lungs due to linearity transfer of cadmium from burning tobacco leaves into smoke [3]. In human body cadmium accumulates mainly in the kidney cortex where its elimination half-time has been estimated at 10–30 years. Concentration of cadmium in urine is proportional to its kidney content therefore reflects integrated past exposure and cadmium body burden [4].

Cadmium is a well-known nephrotoxic agent able to induce renal tubular dysfunction at relatively low exposure level [2]. The first signs of cadmium toxic effect on kidneys are increased excretion of low-molecular-weight proteins and tubular enzymes in urine. Prolonged exposure to high cadmium levels is also related to disturbances in bone metabolism. Several cases of osteomalacia and osteoporosis in combination with kidney damage were reported among exposed to cadmium workers and Japanese women consuming heavily cadmium-polluted rice [5]. The disease characterized multiply fractures and long bone deformation with severe pain for which reason it was called Itai-itai ('ouch-ouch') disease.

Some epidemiological data suggest that low level cadmium exposure may also be associated with cadmium osteotoxicity and increase risk of osteoporosis [2]. It is not clear if this effect is related to cadmium-induced renal dysfunction or possibly direct effect of cadmium to bones exists. Nevertheless, it is well known that incidences of osteoporosis increase in industrialized countries, especially among cigarette smokers what would suggest negative effect of low cadmium exposure on bone metabolism.

The aim of the study was to assess the relation between urinary cadmium and pyridinoline (Pyr) and deoxypyridinoline (DPyr) crosslinks in urine among subjects with only environmental exposure to cadmium. (Pyr) and (DPyr) are the first and specific markers of bone resorption processes. They are synthesized in posttranslational processing of lysine and hydroxylysine residue of collagen and are essen-

tial for stabilizing the mature forms collagen fibers and elastin. During bone resorption Pyr and DPyr are excreted into the circulation through collagen degradation and eliminated with urine, where they can be measured by HPLC method with fluorescence detection. Positive correlations between concentration of cadmium in urine and Pyr and DPyr urinary excretion may indicate significant impact of low cadmium exposure on higher bone resorption. Additionally calcium in urine was determined.

## MATERIAL AND METHODS

The first void urine spot samples were collected from 36 patients (28 women and 7 men) who were examined for possible toxic effects of cadmium exposure. The patients participated in the study following health survey of population who inhabited one of the community in the southern Poland. The local Biomedical Ethics Committee approved the study protocol for this health survey. The study was extended to include possible toxic effects of cadmium exposure in patients with elevated urinary cadmium level. All the examined patients were with no history of metabolic bone diseases and with no signs of tubular kidney dysfunction. In 2010 the level of cadmium in moss (*Brachythecium rutabulum*) originated from the patients residential area were in the range of 0.73–2.16 µg/g. For the reference in the non-polluted area of Sobieszewska Island near Gdańsk we obtained cadmium levels in the range of 0.10–0.27 µg/g whereas and in the Upper Silesian region – one of the most polluted area in Poland – they were in the range of 4.29–28.67 µg/g. Soils cadmium levels accounted 0.46–1.66 µg/g in the patients area which is within the range from increased cadmium content to low cadmium contamination (geometric mean GM in Poland is 0.21 µg/g) [6]. It is recommended that vegetables growing on soils with increased cadmium content should not be designed for children. On low – contaminated soils some vegetables such as lettuce, spinach or cauliflower should not grow, but cereals, root crops and fodder can be cultivated [7].

Cadmium in urine was measured by graphite furnace atomic absorption spectrometry technique with Zeeman background correction system using PerkinElmer 4100ZL instrument (Bodyswerk, Germany). Acidified samples (pH < 2) were diluted in 1:1 ratio with 0.8 M nitric acid and 20 µl of the solution was introduced into graphite tube. Calibration was performed using the method of standard addition using peak area measurements mode. The precision was 1.6–8.3% relative standard deviation at concentration

0.5–7.0 µg/l. The limit of detection was 0.10 µg/l. Urinary cadmium was corrected for urinary creatinine which was determined by Jaffé photometric method. Laboratory regularly participates in the German External Quality Assessment Scheme intercomparison programme for toxicological analyses in biological materials and complies with the requirements for determining cadmium and creatinine in urine. In 2008 for environmental medical field the results for urinary samples with target cadmium levels of 1.04 and 3.41 µg/l were 0.91 and 3.13 µg/l respectively, which was within the accepted ranges. Mean of the results from the first (about 1 year earlier) and the present determination of cadmium in urine were expressed as a mean cadmium concentration.

Pyr and DPyr in urine were measured by HPLC after hydrolysis of urine in 6 M hydrochloric acid. The method was previously described elsewhere [8]. Briefly, samples were mixed with concentrated hydrochloric acid in 1:1 ratio and heat at 120 for 4 h in glass ampoules. The acid hydrolysates were subsequently mixed with butanol-acetic acid-water mixture (4:1:1) and purified using cellulose CF1 (Whatman). The crosslinks were then eluted with water and after evaporation to dryness resuspended in 1% perfluorobutyric acid (HFBA Sigma Aldrich) and measured using ions – pairs chromatography. Lichrospher 100 LichroCART 250-4 RP-18 5 µm chromatographic column was used with AT 1200 high pressure liquid chromatograph (Agilent Technologist, USA) equipped with a set of pumps, thermostat, autosampler and fluorescence detector. The column was equilibrated with 0.01 M HFBA in water (solvent A) 80% and 0.01 M HFBA in acetonitrile: water 3:1 (solvent B) 20%. Elution of the crosslinks was achieved at ambient temperature at a flow-rate 0.8 ml/min in gradient elution: time 0–20 min solvent A 80% linear change to 70%. The column was washed with 100% of the solvent B for 10 min. Fluorescence was monitored using excitation and emission wavelength 295 and 395 nm, respectively. Calibration was performed using external standard contained crosslinks in concentration 1237 pmol/ml for pyr and 432 pmol/ml deoksypridynoline in lyophilized urine (Chromsystems – Crosslinks urine calibration standard). The values of urinary Pyr and DPyr in samples were expressed as per mmol of urinary creatinine.

Calcium in urine was determined using flame atomic absorption spectrometry. Standards and samples were introduced into air-acetylene flame after 50 fold dilution with 1% lanthanum chloride solution.

Data was analyzed using Statistica 9.1 Software. Descriptive statistic with means, standard deviations and ranges was used for results presentation and sub-

ject characterization. Simple regression analysis was performed between markers and the statistic significance of the correlation was determined by Spearman's rank correlation coefficient. To assess effect level we used Kruskal-Wallis test followed by Dunn's *post hoc* tests for categorized cadmium level in urine. P values of less than 0.05 were considered significant.

## RESULTS

The examined physiological and biochemical parameters are showed in Table I. Men participation was far less than women.

**Tab. I.** Participant characteristics and results of biochemical examination (n = 36, females n = 28; males n = 7)

**Tab. I.** Charakterystyka badanej grupy oraz wyniki badań biochemicznych (n = 36, kobiety n = 28, mężczyźni n = 7)

Parameter	Values
Age (year)	
Mean ± SD	58.7 ± 9.9
Median	58
Range	34–77
Height (cm)	
Mean ± SD	163.9 ± 7.2
Median	161
Range	152–178
Weight (kg)	
Mean ± SD	69.7 ± 8.4
Median	69
Range	50–90
BMI (kg/m <sup>2</sup> )	
Mean ± SD	26.1 ± 4.1
Median	26
Range	15.7–37.0
Smoking cigarettes (n)	
never/past/current	25/5/6
Cadmium in urine (µg/g creatinine)	
Mean ± SD	1.63 ± 0.96
Median	1.57
Range	0.29–6.01
Pyr in urine (nmol/mmol creatinine)	
Mean ± SD	43.5 ± 15.1
Median	42.9
Range	16.0–80.2
DPyr in urine (nmol/mmol creatinine)	
Mean ± SD	8.9 ± 3.41
Median	8.8
Range	3.0–16.6
Calcium in urine (mg/g creatinine)	
Mean ± SD	125.6 ± 65.3
Median	117.6
Range	10.2–284.5

The Spearman rank correlation coefficients for bone-related variables and urinary cadmium were showed in Table II. In the investigated group there were significant positive correlation between cadmium levels in urine and Pyr and DPyr excretion. None of the other variables correlated significantly with examined bone resorption markers excluding calcium

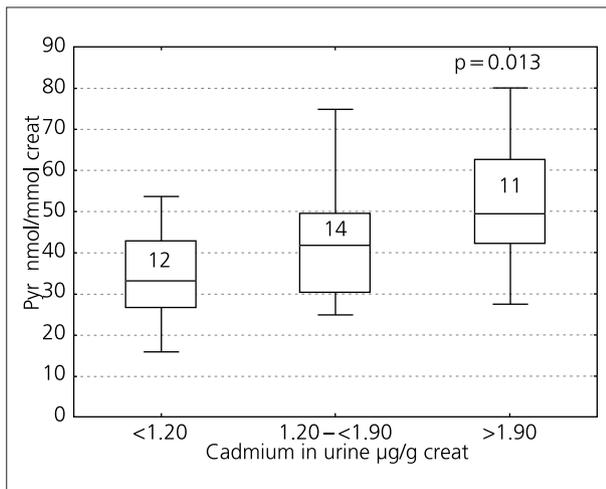
excretion in urine. Excretion of Pyr and DPyr differed significantly between group with the lowest cadmium concentration ( $< 1.2$ ) and group with the highest cadmium concentration ( $> 1.9$ ) what is showed in Figure 1 and 2. In the group with the highest cadmium levels Pyr and DPyr median values increases of 49,8% ( $p=0,012$ ) and 37,5% ( $p=0,049$ ), respectively.

**Tab. II.** Spearman's rank correlation coefficients for the associations between cadmium exposure and expected factors linked to bone metabolism

**Tab. II.** Współczynniki korelacji rang Spearmana dla zależności pomiędzy narażeniem na kadm i przewidywanymi czynnikami związanymi z metabolizmem kości

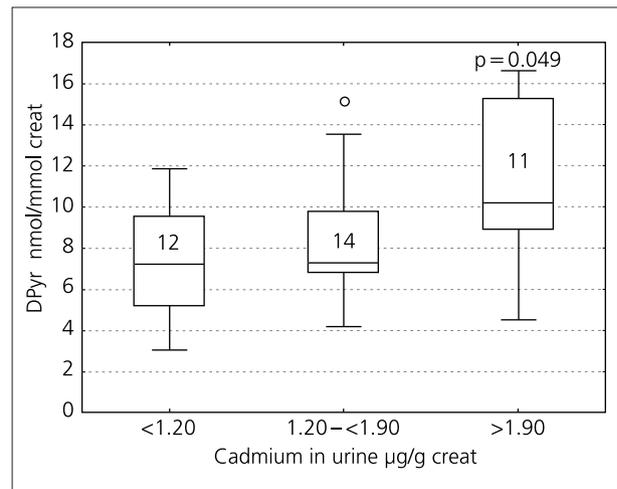
	Cd urine	Cd urine meana	Sex	Years of smoking	Weight	Pyr urine	DPyr urine	Ca urine
Age	0.12	0.03	0.03	-0.09	0.04	0.11	-0.13	-0.04
Sex (0,1)	0.07	0.14						
Years of smoking	0.18	0.19	-0.44*					
Weight	0.31	0.40*	-0.07	0.09				
Pyr urine	0.49*	0.45*	0.07	-0.07	0.28			
DPyr urine	0.34*	0.45*	0.22	0.05	0.32	0.72**		
Ca urine	0.36*	0.36*	0.06	0.18	0.40*	0.46*	0.41*	

\*  $p < 0,05$ ; \*\*  $p < 0,001$ , a – mean value for the present and past (about 1 year earlier) examination



**Fig. 1.** Pyridinoline in urine related to urinary cadmium (categorized) in population study. Boxes depict 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> percentiles and whiskers minimum and maximum. Numbers inside boxes indicate the number of samples. Value for difference between the lowest exposed group and highest exposed group (Kruscal-Wallis test with multiple comparisons)

**Ryc. 1.** Zawartość pyrydynoliny w moczu w stosunku do zawartości kadmu w moczu (wartości skategoryzowane) w badanej populacji. Ramki oznaczają 25, 50 i 75 percentyl, a wąsy minimum i maksimum. Wartość w ramce oznacza ilość próbek. Wartość p dla różnic pomiędzy grupą o najniższym a najwyższym narażeniu (test Kruscala-Wallisa z porównaniami wielokrotnymi)



**Fig. 2.** Deoxypyridinoline in urine related to urinary cadmium (categorized) in population study. Boxes depict 25<sup>th</sup>, 50<sup>th</sup>, 75<sup>th</sup> percentiles and whiskers minimum and maximum, excluding outliers (circle). p Value for difference between the lowest exposed group and highest exposed group (Kruscal-Wallis test with multiple comparisons)

**Ryc. 2.** Zawartość deoksyperydynoliny w moczu w stosunku do zawartości kadmu w moczu (wartości skategoryzowane) w badanej populacji. Ramki oznaczają 25, 50 i 75 percentyl, a wąsy minimum i maksimum z wyłączeniem wartości odstających (okrąg). Wartość w ramce oznacza ilość próbek. Wartość p dla różnic pomiędzy grupą o najniższym a najwyższym narażeniu (test Kruscala-Wallisa z porównaniami wielokrotnymi)

## DISCUSSION

In this study we have observed significant positive correlation between cadmium body burden reflected by Cd concentration in urine and urinary Pyr and DPyr crosslinks excretion. In the last years many of epidemiological studies also indicated cadmium toxic action on bone in low environmental exposure level. Alven et al. found that cadmium dose was inversely related to forearm Bone Mineral Density (BMD) and tubular proteinuria particularly in person over 60 years of age [9]. Studies in China reported that cadmium exposure was related to kidney tubular damage and osteoporosis suggesting possible association between effects on bones and kidneys [10, 11]. However according to their recent finding the effects on kidney showed to be reversible if the exposure to Cd decreased whereas bone effects seems to persist even after exposure cessation [12]. In Japanese study Honda et al. indicated that an index of calcaneal bone mass was inversely correlated with urinary cadmium in the absence of kidney damage [13]. Gallangher et al. found statistically significant association between Cd exposure and osteoporosis and BMD in the US female population > 50 years of age and also suggested direct action of cadmium on bones because of increased odds for osteoporosis at Cd levels below those previously associated with renal tubular dysfunction [14]. In Swedish survey among population with very low environmental cadmium exposure (GM 0.52 µg/L) Akesson et al. found small but clearly association between increasing cadmium body burden and decreasing BMD as also increasing urinary DPyr measured in Pyrilinks-D immunoassay [15]. It was suggested that cadmium decreased bone mineral density through direct osteotoxic effect without earlier cadmium-induced renal tubular dysfunction. This finding was subsequently supported by Belgian study where Pyr and DPyr crosslinks were positively correlated with 24 h-excretion of cadmium in urine [16]. Direct osteotoxic effects of Cd possibly through activation of osteoclasts was also suggested on the base of animal studies [17].

We performed study in a small group of patients with middle exposure level (GM 1.63 µg/g creatinine). Nevertheless, our results are in accordance with finding of cadmium-associated effects on bone-resorption markers. It also suggests direct action of cadmium on bone metabolism with the absence of earlier kidney effects measured as an increase in excretion of low-molecular weight proteins and N-acetylo-β-D-glucosamidase in urine (data not presented). In opposite to these finding other Japanese study conducted by Horiguchi et al. did not reveal

any association between Cd exposure and BMD after adjustment for renal tubular function [18]. Trzcin-ka-Ochocka et al. also reported any contributions of cadmium exposure to decreased BMD. Body weight and additionally age in females and urinary calcium in males were the only factors influencing BMD in the multivariate analysis. It was suggested that this inconsistencies would be attributed to differences between population being examined [19]. In study where the lack of association with cadmium and BMD was reported mainly young women and men were recruited whereas older individuals were examined in other mentioned studies. In our study older subjects were also examined with mean age of 59 years.

According to many previous finding our study also indicates increased calcium excretion with higher cadmium concentration in urine. It is not clear if increased calcium excretion is connected to kidney damage or higher rate of bone resorption. The increased calcium excretion due impaired tubular reabsorption is suggested as a possible mechanism of cadmium effect on bones. However, the findings by Akesson et al. and Shuttle et al. showed that PTH (parathyroid hormone) levels were inversely correlated to Cd exposure suggesting that calcynuria was most likely a consequence of increased bone resorption rather than decreased tubular calcium reabsorption [15, 16]. In this second case increased PTH levels should be expected. Recent study have also revealed that serum 1,25-dihydroxy vitamin D did not correlate with cadmium in urine despite higher markers of tubular damage and lower BMD in high cadmium women group compared to the women in the low-cadmium group [20]. This is in opposite with postulated mechanism of cadmium osteotoxicity by lowering active form of vitamin D due to kidney impairment.

Our simple dose-effect analysis showed the influence of increasing Cd body burden on increased bone resorption markers. None of the other studied variables correlated significantly with Pyr and DPyr excretion in urine. Men exhibited similar levels of crosslinks in urine as women. Alfen et al. found even higher OR for osteoporosis for men than for women in the cadmium dose range of 0.5–3.0 µg/g creatinine [9]. Trzcin-ka-Ochocka et al. also suggested that men may be more sensitive group for the impact of Cd on bone density than women besides higher BMD [19]. After categorization across amount of cadmium excretion the statistically significant increase in Pyr and DPyr concentration could be seen at the highest exposed group with cadmium excretion over 1.9 µg/g creatinine. Recently estimated

benchmark dose with 5% additional risk of osteoporosis in women due to cadmium exposure was 2.9 µg Cd/g creatinine and corresponding lower 95% confidence limit of the benchmark dose was 1.6 µg Cd/g creatinine [21]. Our results seem to be in accordance with this estimation however it should be noted that significantly increased odds ratio for bone effects was observed even at lower exposure levels. The effects were more pronounced for Pyr than for DPyr despite that DPyr is suggested to be more specific marker for bone tissue than Pyr. This observation is consistent with findings reported by Schutte et al. which also indicated that Pyr is stronger associated with cadmium urinary excretion than DPyr [16]. To avoid spurious results they used 24-hour excretion of cadmium with urine. We used creatinine corrected cadmium, but mean cadmium levels from two determinations also correlated significantly with markers of bone resorption.

Unexpectedly cadmium in urine was not correlated to smoking years and smoking status suggesting other important sources of exposure in the examined subjects. It is also possible that method based on questionnaire only and not use more objective method for smoking status assessment as for example determination of cotinine concentration in urine may be a reason for the lack of such associations. However, following Swedish studies on fracture incidents conducted among men and women cadmium osteotoxicity is independent from smoking and especially evident in never-smokers, which are exposed to cadmium mainly via the foods [22, 23].

## CONCLUSIONS

We conclude that long-term cadmium exposure may increase bone resorption at relatively low environmental exposure level. This association suggests that cadmium contributes to bone metabolism and may increase risk for development of osteoporosis and occurrence of fracture incidents. Since cadmium is a widespread contaminant and exposure is common especially via the foods intensive action toward decreasing exposure level seems to be strongly recommended.

### Founding

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