

Assessment of urinary protein 1 and transferrin as early markers of cadmium nephrotoxicity

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Abstract

Transferrin and protein 1, a sex linked α_2 -microprotein, were assayed in urine from 58 workers exposed to cadmium (Cd) in a non-ferrous smelter and from 58 age matched referents. These two new markers of nephrotoxicity were compared with urinary β_2 -microglobulin (β_2 -m), retinol binding protein (RBP), albumin, and β -N-acetylglucosaminidase (NAG). The response of protein 1 to Cd tubulotoxicity was similar to that of β_2 -m, RBP, and NAG. In Cd workers, protein 1 had a correlation with urinary Cd ($r = 0.56$) similar to β_2 -m ($r = 0.48$), RBP ($r = 0.58$), and NAG ($r = 0.49$). Values of these three low molecular weight proteins and of NAG were increased only in workers with urinary Cd higher than $10 \mu\text{g/g}$ creatinine. Urinary transferrin and albumin were similarly affected by exposure to Cd. Their response, however, was clearly more sensitive than that of low molecular weight proteins. Prevalences of positive values of these two high molecular weight proteins were not only higher but also tended to rise at lower concentrations of Cd in urine or blood. This finding suggests that in some subjects subtle defects in glomerular barrier function may precede the onset of proximal tubular impairment after chronic exposure to Cd. It remains to be assessed whether these subjects are more at risk of developing renal insufficiency.

The early nephrotoxic action of cadmium (Cd) in man can be detected on the basis of several urinary or bloodborne markers.¹⁻³ Tests that are currently recommended for the health surveillance of populations exposed to Cd rely on the measurement of

urinary proteins which reflect the functional integrity of the tubule or the glomerulus according to their size. Low molecular weight proteins such as β_2 -microglobulin (β_2 -m),⁴ retinol binding protein (RBP),^{5,6} or α_1 -microglobulin⁷ are currently used for screening for proximal tubular injury whereas the assay in urine of a high molecular weight protein such as albumin permits the assessment of glomerular filter selectivity, at least before tubular function is compromised.^{8,9} According to recent studies the urinary activity of β -N-acetylglucosaminidase (NAG) is also a sensitive indicator of excessive absorption of Cd.^{10,11}

In this study we have assessed two new potential markers of incipient Cd nephropathy, urinary protein 1 and transferrin. Protein 1 is an α -microprotein (molecular weight 20 000) which may be the equivalent in man of the androgen dependent rat α_2 -globulin.¹² It is a sex linked protein that is excreted in greater amounts in the urine from men after puberty. The origin of urinary protein 1 is unknown but the data we have so far collected suggest that it might constitute a new marker of impaired tubular reabsorption.¹²

Urinary transferrin was determined because recent studies suggest that it may be a more sensitive index of glomerular barrier defect than the microalbuminuria test.^{13,14} These two markers have been assayed in the urine from workers exposed to Cd in a non-ferrous smelter and compared with urinary β_2 -m, RBP, albumin, and NAG. The relation between these biochemical markers and indicators of exposure to Cd was also examined.

Materials and methods

STUDY POPULATIONS

The study was conducted on 58 male workers exposed to Cd in a non-ferrous smelter. The control group consisted of 58 age matched men recruited from a factory with no risk of exposure to Cd or other heavy metals. None of the exposed workers or referent subjects was occupationally exposed to lead or mercury as shown by the concentrations of these metals in blood ($< 35 \mu\text{g}/100 \text{ ml}$) and urine ($< 5 \mu\text{g/g}$ creatinine). The characteristics of the two populations are summarised in table 1.

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Table 1 Characteristics of control and cadmium exposed workers

	Control workers (n = 58)	Cadmium workers (n = 58)	p value†
Age (y)*	43.1 (10.4)	43.5 (10.5)	NS
Duration of exposure (y)*	—	10.4 (6.6)	—
Cd concentration in blood ($\mu\text{g/l}$)†	0.89 (0.3–2.9)	6.54 (1.6–51)	<0.001
Cd concentration in urine ($\mu\text{g/g}$ creatinine)†	0.66 (0.14–2.5)	6.23 (0.87–165)	<0.001

*Arithmetic mean (SD).

†Geometric mean (range).

‡Student's *t* test; NS *p* > 0.05.

COLLECTION OF BIOLOGICAL SAMPLES

A spot urine sample was collected from each subject and 4.5 ml were immediately transferred to a tube containing 0.4 ml of a 1 M phosphate buffer (pH 7.6) containing 0.2% sodium azide. A sample of venous blood was also taken and immediately separated into two portions, one for metals analysis (on an EDTA tube) and the other for serum separation. All samples were collected in metal free tubes or bottles and stored at 4°C until determination of protein.

BIOLOGICAL ANALYSES

Urinary creatinine was measured by Jaffé's method.¹⁵ The concentrations of protein 1, transferrin, albumin, RBP, and β_2 -m were determined by an automated assay relying on latex particle agglutination.¹⁶ All the antibodies were obtained from Dakopatts, Denmark. The assay of protein 1 was calibrated with a batch of purified antigen provided by Dakopatts. Standards for the determination of albumin and transferrin were obtained from Sigma Chemical Co (St Louis, Missouri) whereas that for β_2 -m and RBP consisted of proteins purified in our laboratory as described previously.¹⁷

The detection limits of these immunoassays in urine (10-fold dilution) were 5 $\mu\text{g/l}$ for β_2 -m and transferrin, 10 $\mu\text{g/l}$ for protein 1 and RBP, and 50 $\mu\text{g/l}$ for albumin. The activity of NAG in urine was measured by the automated fluorimetric assay described by Tucker *et al.*¹⁸

The determinations of Cd and lead in whole blood or urine were performed by electrothermal atomic absorption spectrometry (Perkin Elmer Zeeman

3030) using the stabilised temperature platform furnace coupled with a Zeeman effect background correction system. Total mercury in urine was analysed using an automated cold vapour atomic absorption technique.¹⁹

STATISTICS

Parametric tests (Student's *t* test, Pearson's correlation) were applied on log transformed data for all biological parameters except serum β_2 -m. Prevalences of raised values were compared by 2×2 χ^2 test with Yates' correction. These prevalences of abnormal values were calculated by using as a normal upper limit the mean (geometric except for serum β_2 -m) plus two standard deviations of the results found in the control group. For serum β_2 -m and urinary β_2 -m, RBP, and albumin we also calculated the prevalence of abnormal results on the basis of the upper normal limits routinely used in our laboratory.

Results

Compared with the age matched referents, workers exposed to Cd had significantly higher concentrations of β_2 -m in serum and of β_2 -m, transferrin, albumin, and higher NAG activity in urine. The mean values for urinary protein 1 and RBP did not differ between the control and Cd groups (table 2).

The prevalences of raised values of all biochemical parameters of nephrotoxicity were increased in the Cd group (table 3). The difference between the control and Cd groups, however, was significant only for the urinary output of albumin and transferrin.

Table 2 Indicators of nephrotoxicity in control and cadmium workers

	Control workers	Cadmium workers	p value‡
Serum β_2 -m concentration (mg/l)*	1.4 (0.43)	1.81 (0.53)	<0.001
Urinary β_2 -m concentration ($\mu\text{g/g}$ creatinine)†	53.3 (4.7–255)	107 (11.7–136 000)	<0.005
Urinary RBP concentration ($\mu\text{g/g}$ creatinine)†	66.4 (14–378)	78 (20–73 000)	NS
Urinary protein 1 concentration ($\mu\text{g/g}$ creatinine)†	92.7 (14–845)	107 (17.3–16 500)	NS
Urinary albumin concentration (mg/g creatinine)†	5.6 (2.3–25.5)	8.8 (2.6–331)	<0.005
Urinary transferrin concentration ($\mu\text{g/g}$ cr)†	194 (50–1710)	390 (65–15 700)	<0.001
Urinary NAG activity (U/g creatinine)†	0.96 (0.18–2.84)	1.3 (0.46–8.76)	<0.005

*Arithmetic mean (SD).

†Geometric mean (range).

‡Student's *t* test; NS, *p* > 0.05.

Table 3 Prevalence of increased values of nephrotoxicity markers in control and cadmium groups

	Control group		Cd group		p value†
	No	%	No	%	
Serum β_2 -m	2*	3.4	9	15.5	NS
	2†	3.4	6	10.3	NS
Urinary β_2 -m	0*	0	4	6.9	NS
	0†	0	5	8.6	NS
Urinary RBP	2*	3.4	5	8.6	NS
	1†	1.7	4	6.9	NS
Urinary protein 1	1	1.7	4	6.9	NS
Urinary albumin	3*	5.2	13	22.4	< 0.025
	2†	3.4	11	19.0	< 0.025
Urinary transferrin	2	3.4	13	22.4	< 0.01
Urinary NAG	1*	1.7	6	10.3	NS

*The values higher than the mean + 2 SD of the values (normal or log transformed) of the control group (2.26 mg/l for serum β_2 -m, 2.7 U/g creatinine for urinary NAG, and 0.324, 0.24, 0.607, 14.8, and 0.87 mg/g creatinine for urinary β_2 -m, RBP, protein 1, albumin and transferrin) are classified as positive.

†The values higher than the upper limits of normal used in the laboratory (2.4 mg/l for serum β_2 -m, 20 mg/g creatinine for albumin, and 0.3 mg/g creatinine for both urinary β_2 -m and RBP) are classified as positive.

‡ χ^2 test; NS, $p > 0.05$.

In this population of workers the response of urinary proteins of high molecular weight to Cd exposure was thus clearly more sensitive than that of low molecular weight proteins. Calculations with the upper limits of normal values routinely used in our laboratory or the 95th percentiles of the values found in the present control group led to the same conclusion (table 3).

In the group of Cd workers the seven markers of nephrotoxicity were significantly correlated with the internal dose of Cd, assessed on the basis of urinary Cd (figs 1 and 2) or the Cd concentration in blood (results not shown). Urinary protein 1 was as well correlated with urinary Cd as β_2 -m, RBP, or NAG. The weakest correlation was that between the urinary concentration of transferrin and the concentration of Cd. The relation between urinary albumin and Cd in exposed workers was noteworthy: it was as high as that between β_2 -m and Cd.

Dose response relations were established by combining referents and exposed workers and by dividing the total population into four classes according to the concentration of Cd in urine (fig 3) or in blood (fig 4).

For all the measured parameters, prevalences of abnormally raised values were significantly increased when the concentrations of Cd in urine or blood exceeded 10 $\mu\text{g/g}$ creatinine in urine and 10 $\mu\text{g/l}$ in blood (the values that are presently considered as critical for the development of Cd nephropathy).^{8,19-26} Prevalence of raised values for low molecular weight proteins showed no tendency to increase before these critical concentrations were reached. In some subjects, however, the serum concentration of β_2 -m,

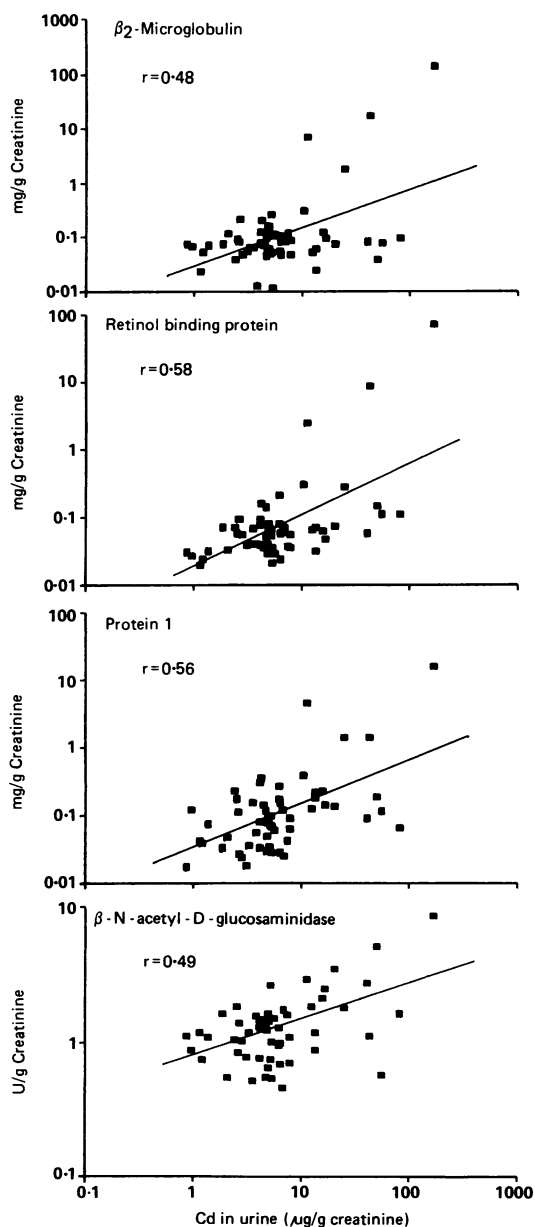


Figure 1 Correlations between urinary excretion of β_2 -microglobulin, retinol binding protein, protein 1, NAG, and that of Cd in 58 Cd smelter workers ($p < 0.001$).

and the urinary output of albumin and transferrin tended to become abnormal at a lower internal dose of Cd. This phenomenon could not be ascribed to an age effect since the subgroups were well matched for age.

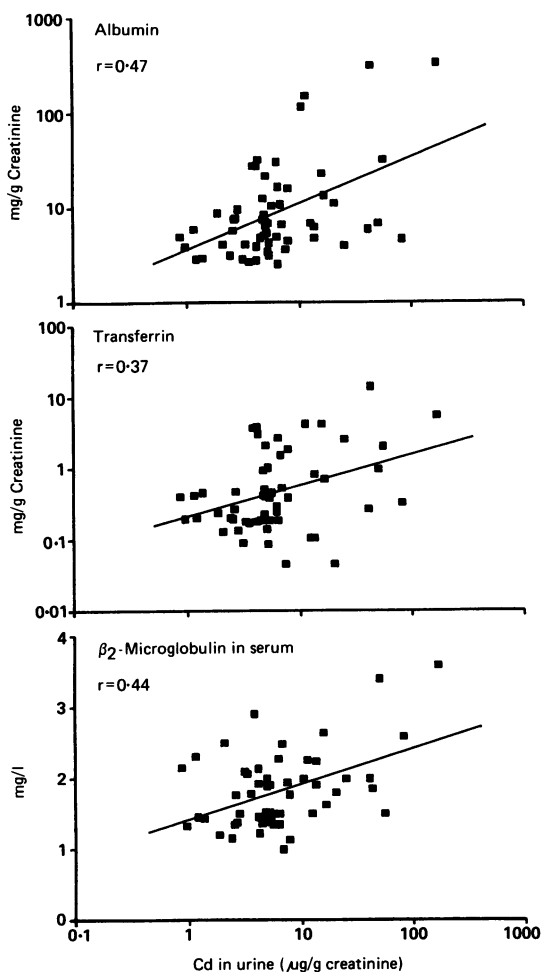


Figure 2 Correlations between albuminuria, transferrinuria, and serum concentration of β_2 -m, and urinary excretion of Cd in 58 Cd smelter workers ($p < 0.005$).

Discussion

The present study indicates that the behaviour of protein 1 in the urine of Cd workers is similar to that of β_2 -m, RBP, and presumably other low molecular weight proteins. A feature of protein 1 which may be of interest for screening purposes is its extraordinary stability in urine, which is much superior to that of β_2 -m and even RBP. Protein 1 is stable in a pool of normal urine incubated at pH 4 and 37°C for 24 h. Also, no degradation was found in urine samples that had been stored for six months at 4°C (A Bernard and colleagues, unpublished observation). The origin and biological function of this new protein are still unknown and little information is presently available

about the physiological or pathological processes that may affect its concentration in biological fluids.¹² The sex dependency of urinary protein 1 suggests that its synthesis is controlled by hormonal factors, in particular, androgens.

By contrast with the studies of Kawada *et al*¹⁰ and Chia *et al*,¹⁷, our data do not indicate that urinary NAG activity is a more sensitive indicator of renal tubular effects than β_2 -m concentrations. Evidently the urinary activity of NAG shows no greater tendency to become abnormal at lower concentrations of Cd in blood and urine than β_2 -m or other markers of proximal tubule injury. Nor does activity of this enzyme correlate better with the internal dose of Cd than do other indicators of nephrotoxicity. A possible explanation for these discrepancies might be in the matching between exposed and control workers, which was not perfectly achieved with respect to age in the study by Kawada *et al*,¹⁰ and in the study by Chia *et al*¹¹ there were 65 exposed and only nine control workers.

It has been known for a long time that the urinary excretion of transferrin parallels that of albumin in patients with enhanced glomerular leakiness.²⁵ The recent introduction of sensitive immunoassays for comparing both proteins has shown, however, that they may respond differently to the early alterations of incipient glomerulopathies. In diabetic patients, urinary transferrin concentration proved to be a more sensitive index of early glomerular involvement than the determination of microalbuminuria.^{13,14} In the population of Cd workers examined here urinary transferrin concentration does not seem to detect the glomerular dysfunction induced by Cd at an earlier stage than albuminuria. This lack of an overt difference between both parameters is probably due to the fact that by contrast with diabetic nephropathy, which primarily affects the glomeruli, Cd nephropathy may affect both the glomeruli and the tubules. In most workers with Cd concentration in urine higher than 10 $\mu\text{g/g}$ creatinine the increased loss of albumin and transferrin in urine is accompanied by an enhanced urinary excretion of low molecular weight proteins. In these workers the comparison of urinary albumin and transferrin concentrations as indices of the glomerular filter selectivity is obviously confounded by the concurrent tubular impairment which can also cause a rise in high molecular weight proteins in urine.

By contrast, the high molecular weight proteinuria found in workers with urinary Cd concentrations between two and 10 $\mu\text{g/g}$ creatinine was not associated with signs of tubular damage. Seven workers were positive on the basis of the albuminuria test and six on the basis of the transferrinuria assay but none had an abnormal urinary excretion of β_2 -m or RBP (the highest values were 212 μg β_2 -m and 214 μg RBP/g creatinine). The albuminuria of these

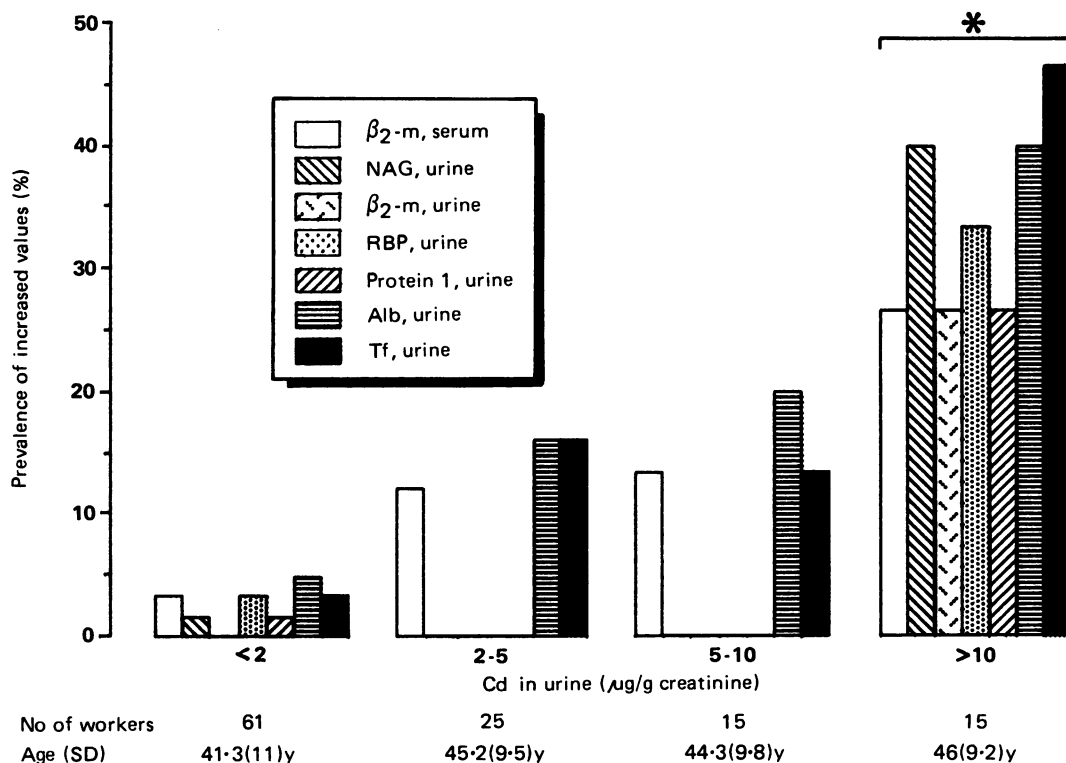


Figure 3 Prevalences of raised values of markers of nephrotoxicity as a function of urinary Cd in Cd smelter workers and their referents. Prevalences were established on basis of upper limits of normal defined in control group and given in table 3 footnote a.

*Statistically different from controls ($2 \times 2 \chi^2$ test).

workers was slight and below the threshold of detection by conventional dipstick tests. Nevertheless, if the criterion for diagnosing the presence of microalbuminuria in diabetic patients was applied (30 mg/day or 20 mg/g creatinine)²⁶ five must be considered as microalbuminuric. Although the numbers of Cd workers presenting with an enhanced albuminuria or transferrinuria without change in low molecular weight urinary protein excretion are practically the same, it is worth noting that on average, transferrinuria in these subjects was increased by a factor of 11 against a factor only half that for albuminuria.

The occurrence of a slight microalbuminuria or microtransferrinuria in Cd workers at exposure concentrations inferior to those considered currently as critical deserves special attention. Previous studies have reported that the rise of albuminuria in Cd exposed workers occurs concomitantly with or after the occurrence of proximal tubular dysfunction.^{3 9 20 27 28} It should be emphasised, however, that in these studies albumin and other high molecular

weight proteins were determined in urine by immunoprecipitation techniques (electroimmunoassay, nephelometry, or radial immunodiffusion) that are much less sensitive than most immunoassays presently used to detect microalbuminuria. The possibility that a slight increase in albumin excretion had been overlooked in these earlier studies cannot be excluded. Further studies are required to determine whether, as suggested by our data, subtle alterations of glomerular barrier selectivity may precede the onset of proximal tubular dysfunction in some workers chronically exposed to Cd and whether, as shown in diabetic patients, an isolated increased urinary excretion of high molecular weight protein after chronic exposure to Cd is predictive of an increased risk of renal insufficiency.

The finding in some workers (particularly among those with the highest internal dose of Cd) of increased serum β_2 -m concentrations suggests a reduction in the glomerular filtration rate. As noted by other investigators, however, raised concentrations of β_2 -m in serum are not always associated with

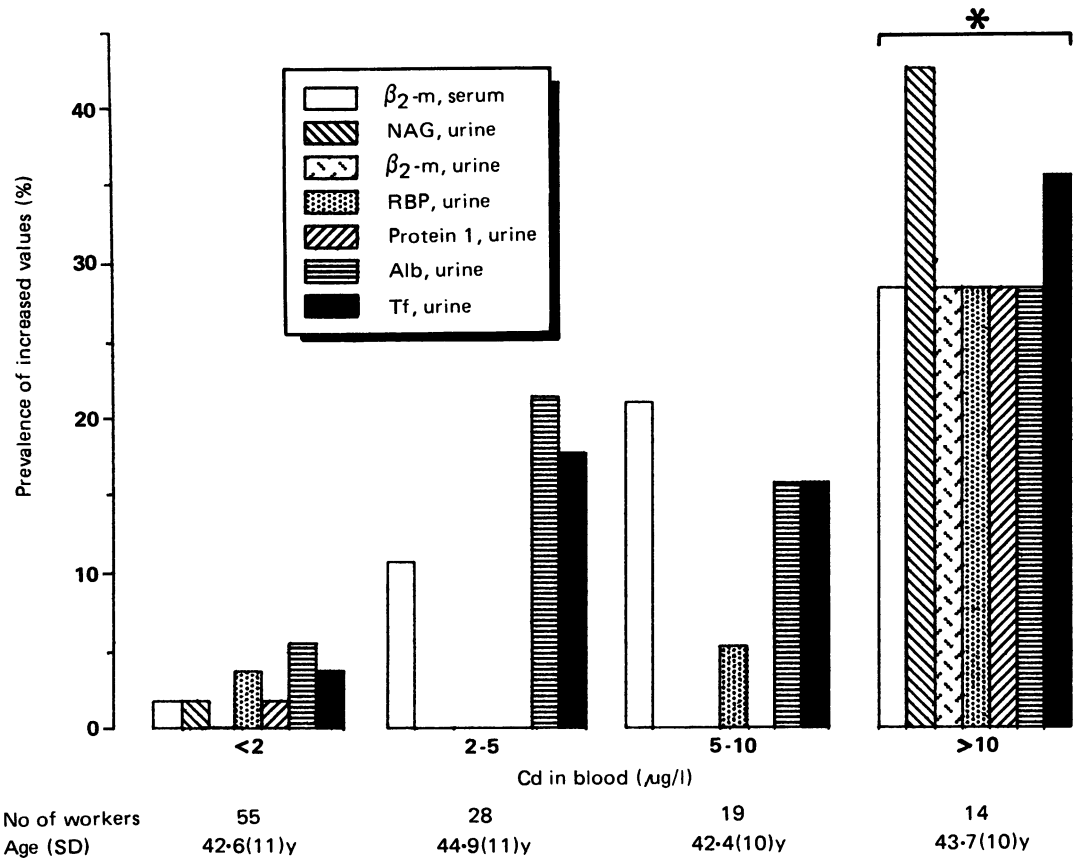


Figure 4 Prevalences of raised values of markers of nephrotoxicity as a function of the Cd concentration in blood in Cd smelter workers and their referents. Prevalences were established on basis of upper limits of normal defined in control group and given in table 3 footnote a.

*Statistically different from controls ($2 \times 2 \chi^2$ test).

a low or a high molecular weight microproteinuria.^{4,29} Of the seven workers with raised serum $\beta_2</math>-m concentrations (>2.4 mg/l), three showed no disturbances in the urinary excretion of specific proteins. Whether in these subjects the rise in serum $\beta_2</math>-m is caused by a reduction in the glomerular filtration rate or an enhanced synthesis of the protein³⁰ also deserves further study.$$

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