

Article

# The Source and Pathophysiologic Significance of Excreted Cadmium

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**Abstract:** In theory, the identification of the source of excreted cadmium (Cd) might elucidate the pathogenesis of Cd-induced chronic kidney disease (CKD). With that possibility in mind, we studied Thai subjects with low, moderate, and high Cd exposure. We measured urine concentrations of Cd, ( $[Cd]_u$ ); N-acetyl- $\beta$ -D-glucosaminidase, a marker of cellular damage ( $[NAG]_u$ ); and  $\beta_2$ -microglobulin, an indicator of reabsorptive dysfunction ( $[\beta_2MG]_u$ ). To relate excretion rates of these substances to existing nephron mass, we normalized the rates to creatinine clearance, an approximation of the glomerular filtration rate (GFR) ( $E_{Cd}/C_{cr}$ ,  $E_{NAG}/C_{cr}$ , and  $E_{\beta_2MG}/C_{cr}$ ). To link the loss of intact nephrons to Cd-induced tubular injury, we examined linear and quadratic regressions of estimated GFR (eGFR) on  $E_{Cd}/C_{cr}$ , eGFR on  $E_{NAG}/C_{cr}$ , and  $E_{NAG}/C_{cr}$  on  $E_{Cd}/C_{cr}$ . Estimated GFR varied inversely with both ratios, and  $E_{NAG}/C_{cr}$  varied directly with  $E_{Cd}/C_{cr}$ . Linear and quadratic regressions of  $E_{\beta_2MG}/C_{cr}$  on  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$  were significant in moderate and high Cd-exposure groups. The association of  $E_{NAG}/C_{cr}$  with  $E_{Cd}/C_{cr}$  implies that both ratios depicted cellular damage per surviving nephron. Consequently, we infer that excreted Cd emanated from injured tubular cells, and we attribute the reduction of eGFR to the injury. We suggest that  $E_{Cd}/C_{cr}$ ,  $E_{NAG}/C_{cr}$ , and eGFR were associated with one another because each parameter was determined by the tubular burden of Cd.

**Keywords:**  $\beta_2$ -microglobulin; cadmium; creatinine clearance; glomerular filtration; N-acetyl- $\beta$ -D-glucosaminidase; nephron mass; nephrotoxicity

## 1. Introduction

Cadmium (Cd), a divalent metal used for industrial purposes, is an important environmental pollutant in some regions of the world [1–5]. The metal is conveyed to humans in food, air, and tobacco smoke, and subsequently gains access to the circulation through the gut and lungs [5]. Salts of ionized Cd are absorbed in the duodenum; in addition, complexes of Cd with plant metallothioneins (MT) and phytochelatins (PC) may be absorbed in the colon after liberation by bacteria [6]. In the bloodstream,

Cd is bound to red blood cells, albumin, glutathione (GSH), sulfur-containing amino acids, MT, and PC [6–10]. In the liver, hepatocytes take up Cd not bound to MT [10], synthesize MT in response to the metal, and store complexes of CdMT. These complexes are subsequently released from hepatocytes and transported to the kidneys [8,11,12]. Cd in plasma is filterable by glomeruli if it is bound to GSH, amino acids, MT, or PC [8,9], but the fraction of circulating Cd that enters the filtrate is unknown. The proximal tubule reabsorbs and retains most or all of the filtered Cd with an array of channels, solute carriers, and mediators of endocytosis [7,10,13–16]. Basolateral uptake may also add to the cellular content of Cd in the proximal tubule [16–18].

It is currently assumed that the magnitude of a gradually acquired burden determines the toxicity of Cd in tubular cells [19]. The emergence of Cd from lysosomes induces robust intracellular synthesis of MT, which greatly mitigates the injury inflicted by free Cd through complexation of the metal [20]. Nevertheless, a fraction of Cd remains unbound to MT and is presumed to promote autophagy, apoptosis, and necrosis as accumulation of Cd progresses [19,21]. Manifestations of renal toxicity include increased excretion of cellular proteins, impaired reabsorption of filtered substances, histologically demonstrable tissue injury, loss of intact nephrons, and reduction of the glomerular filtration rate (GFR) [5,8,21–27]. GFR may continue to fall for many years after exogenous exposure ceases [22,24], presumably because traffic of CdMT from the liver to kidneys persists.

In human studies of Cd-induced nephropathy, the most commonly assayed marker of tubular cell damage is the lysosomal enzyme N-acetyl- $\beta$ -D-glucosaminidase (NAG). Because NAG is too large to be filtered by glomeruli, excessive excretion ( $E_{\text{NAG}}$ ) signifies tubular injury [28]. The most commonly measured indicator of impaired reabsorption is  $\beta_2$ -microglobulin ( $\beta_2\text{MG}$ ). This small circulating protein is extremely filterable by glomeruli [29]; ordinarily, more than 99% of filtered  $\beta_2\text{MG}$  is reabsorbed [30], but that percentage falls early in the course of tubular injury [31].

Although the urinary excretion rate of Cd ( $E_{\text{Cd}}$ ) is believed to reflect the body burden of the metal [5], the precise source and pathophysiologic significance of excreted Cd have not been clarified. One possibility is that Cd is excreted because it is filtered and not reabsorbed; an alternate possibility is that excretion reflects liberation of Cd into filtrate from injured or dying tubular cells [32]. This distinction is important because the source of urinary Cd is central to the relationship between Cd accumulation and progression of chronic kidney disease (CKD). Herein, we present evidence that excreted Cd emanates from cells that it has injured. The injury leads to the loss of intact nephrons, reduction of GFR, and impaired reabsorption of filtered  $\beta_2\text{MG}$ .

## 2. Materials and Methods

### 2.1. Study Subjects

The Institutional Ethical Committees of Chulalongkorn University, Chiang Mai University and the Mae Sot Hospital approved the study protocol (Approval No. 142/2544, 5 October 2001) [33]. All participants gave informed consent before participation. Subjects were recruited from urban communities in Bangkok in 2001/2002 and from subsistence farming areas in Mae Sot District, Tak Province, Thailand in 2004/2005 [33]. They had lived at their current addresses for at least 30 years. Exclusion criteria were pregnancy, breast-feeding, a history of metal work, and a hospital record or physician's diagnosis of an advanced chronic disease. Because occupational exposure was an exclusion criterion, we presumed that all participants had acquired Cd from the environment.

Cd exposure was low in Bangkok and moderate or high in Mae Sot [33,34]. Determination of exposure was based on reported levels of Cd in rice grains grown in the Cd-affected areas of the Mae Sot District [35,36]. After exclusion of subjects with incomplete datasets, we studied 172, 310, and 222 persons from the low, moderate, and high exposure areas, respectively.

## 2.2. Collection of Biological Specimens and Laboratory Analyses

Second morning-void urine samples were collected after an overnight fast. Within three hours after urine sampling, specimens of whole blood were obtained and serum samples were prepared. Aliquots of urine, whole blood, and serum were transported on ice from a mobile clinic to a laboratory and stored at  $-20\text{ }^{\circ}\text{C}$  or  $-80\text{ }^{\circ}\text{C}$  for later analysis. The assay for urine and serum creatinine concentrations ( $[\text{cr}]_{\text{u}}$ ,  $[\text{cr}]_{\text{p}}$ ) was based on the Jaffe reaction. The urine NAG assay was based on colorimetry (NAG test kit, Shionogi Pharmaceuticals, Sapporo, Japan). The urine  $\beta_2\text{MG}$  assay was based on the latex immunoagglutination method (LX test, Eiken 2MGII; Eiken and Shionogi Co., Tokyo, Japan). When the urine concentration of  $\beta_2\text{MG}$  ( $[\beta_2\text{MG}]_{\text{u}}$ ) was below the limit of detection (LOD),  $0.5\text{ }\mu\text{g/L}$ , the value assigned to  $[\beta_2\text{MG}]_{\text{u}}$  was  $\text{LOD}/(\text{square root of } 2)$ .

For the Bangkok group,  $[\text{Cd}]_{\text{u}}$  was determined by inductively-coupled plasma mass spectrometry (ICP/MS, Agilent 7500, Agilent Technologies), because it had the high sensitivity required to measure Cd concentrations below the detectable limit of atomic absorption spectrophotometry. Multi-element standards (EM Science, EM Industries, Inc., Newark, NJ, USA) were used to calibrate Cd analyses. The accuracy and precision of those analyses were evaluated with reference urine (Lyphochek<sup>®</sup>, Bio-Rad, Sydney, Australia). When  $[\text{Cd}]_{\text{u}}$  was less than the detection limit of  $0.05\text{ }\mu\text{g/L}$ , the concentration assigned was the detection limit divided by the square root of 2.

For the Mae Sot groups,  $[\text{Cd}]_{\text{u}}$  was determined by atomic absorption spectrophotometry (Shimadzu Model AA-6300, Kyoto, Japan). Urine standard reference material No. 2670 (National Institute of Standards, Washington, DC, USA) was used for quality assurance and control purposes. None of the urine samples from the Mae Sot groups were found to have  $[\text{Cd}]_{\text{u}}$  below the detection limit.

## 2.3. Estimated Glomerular Filtration Rate (eGFR)

The glomerular filtration rate was estimated with equations from the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [37,38]. CKD stages 1, 2, 3, 4, and 5 corresponded to eGFR of 90–119, 60–89, 30–59, 15–29, and  $<15\text{ mL/min}/1.73\text{ m}^2$ , respectively. For dichotomous comparisons, CKD was defined as  $\text{eGFR} < 60\text{ mL/min}/1.73\text{ m}^2$ .

## 2.4. Normalization of Excretion Rates to Creatinine Clearance ( $C_{\text{cr}}$ )

Excretion rates of Cd, NAG, and  $\beta_2\text{MG}$  were normalized to  $C_{\text{cr}}$  to yield the ratios  $E_{\text{Cd}}/C_{\text{cr}}$ ,  $E_{\text{NAG}}/C_{\text{cr}}$ , and  $E_{\beta_2\text{MG}}/C_{\text{cr}}$  in units of mass (amount excreted) per volume of filtrate. For  $x = \text{Cd}$ , NAG, or  $\beta_2\text{MG}$ ,  $E_x/C_{\text{cr}}$  was calculated as  $[x]_{\text{u}}[\text{cr}]_{\text{p}}/[\text{cr}]_{\text{u}}$  ([39]; Supplementary Materials).

## 2.5. Statistical Analysis

Data were analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). Distributions of excretion rates for Cd, NAG, and  $\beta_2\text{MG}$  were examined for skewness, and those showing rightward skewing were subjected to base-10 logarithmic transformation before analysis. Departure of a given variable from normal distribution was assessed with the one-sample Kolmogorov–Smirnov test. For continuous variables not conforming to a normal distribution, the Kruskal–Wallis test was used to determine differences among the three localities in  $E_{\text{Cd}}/C_{\text{cr}}$ ,  $E_{\text{NAG}}/C_{\text{cr}}$ ,  $E_{\beta_2\text{MG}}/C_{\text{cr}}$ , and other parameters. The Mann–Whitney  $U$ -test was used to compare mean differences between two groups. The Chi-Square test was used to determine differences in percentage and prevalence data.  $p$ -values  $\leq 0.05$  for two-tailed tests were assumed to indicate statistical significance.

Polynomial regression was used to fit lines and curves to the scatterplots of five pairs of variables, including eGFR versus  $E_{\text{Cd}}/C_{\text{cr}}$ , eGFR versus  $E_{\text{NAG}}/C_{\text{cr}}$ ,  $E_{\text{NAG}}/C_{\text{cr}}$  versus  $E_{\text{Cd}}/C_{\text{cr}}$ ,  $E_{\beta_2\text{MG}}/C_{\text{cr}}$  versus  $E_{\text{Cd}}/C_{\text{cr}}$ , and  $E_{\text{NAG}}/C_{\text{cr}}$  versus  $E_{\beta_2\text{MG}}/C_{\text{cr}}$ . A linear model,  $y = a + bx$ , was adopted if the relationship was monotonic. A quadratic model (second-order polynomial),  $y = a + b_1x + b_2x^2$ , was used if there was a significant change in the direction of the slope ( $b_1$  to  $b_2$ ) for prediction of the dependent variable  $y$ . In both types of equations,  $a$  represented the  $y$ -intercept.

The relationships between  $x$  and  $y$  were assessed with  $R^2$  (the coefficient of determination) and with unstandardized and standardized  $\beta$  coefficients. In linear and quadratic models,  $R^2$  is the fraction of variation in  $y$  that is explained by the variation in  $x$ . In linear models, the unstandardized  $\beta$  coefficient is the slope of the linear regression, and the standardized  $\beta$  coefficient indicates the strength of the association between  $y$  and  $x$  on a uniform scale. To examine quadratic curves relating eGFR to  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$ , we performed slope change analyses with a linear regression method.

### 3. Results

Table 1 presents data concerning age, gender, blood pressure, smoking status, and renal function of subjects with low, moderate, and high environmental exposure to Cd. There were significant differences in age and percentages of women and smokers across the three exposure subsets. Female gender was overrepresented in the moderate exposure group. More than half of subjects in the high exposure group were smokers. Blood pressures were recorded in the low and moderate exposure groups; systolic and mean pressures were significantly higher in the latter.

**Table 1.** Study subjects drawn from three localities.

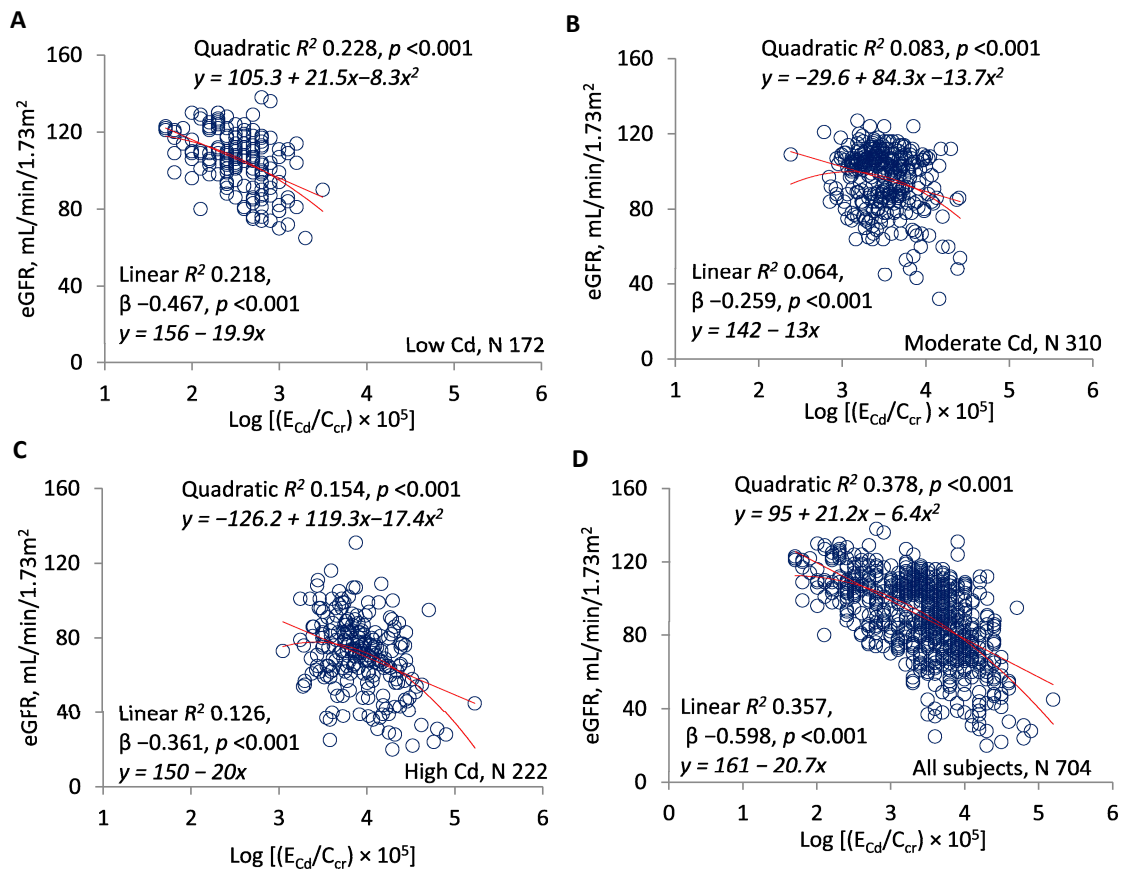
Descriptors	All Subjects	Locality		
		Low Cd	Moderate Cd	High Cd
Number of subjects	704	172	310	222
Women (%)	60.7	47.7	72.9	53.6 *
Smoking (%)	43.6	23.8	40.6	63.1 *
Age (years)	48.34 ± 11.11	38.72 ± 10.29	47.24 ± 4.72	57.34 ± 11.13 †
SBP (mmHg)	122.7 ± 13.5	120.1 ± 10.4	124.2 ± 14.7 ¶	–
DBP (mmHg)	78.9 ± 9.5	78.1 ± 7.6	79.4 ± 10.4	–
MBP (mmHg)	93.5 ± 9.9	92.1 ± 7.9	94.3 ± 3.3 ¶¶	–
eGFR (mL/min/1.73 m <sup>2</sup> )	90.32 ± 21.84	105.46 ± 15.05	95.72 ± 16.13	71.05 ± 19.65 †
CKD prevalence (%)	9.5	0	3.5	25.2
Kidney disease stage (%)				
Stage 1	54.8	84.1	66.5	16.2 *
Stage 2	36.6	15.9	31.0	59.9 *
Stage 3	7.7	0	2.6	20.7 **
Stage 4	1.0	0	0	3.2
Serum creatinine (mg/dL)	0.80 (0.70, 1.0)	0.8 (0.7, 0.9)	0.8 (0.7, 0.9)	1.0 (0.9, 1.2) †
Urine creatinine (mg/dL)	97 (53, 156)	52 (32, 106)	110 (62, 171)	115 (69, 158) †
Urine Cd (µg/L)	3.7 (1.1, 8.3)	0.2 (0.1, 0.6)	3.9 (2.4, 7.2)	8.3 (4.7, 13.9) †
Urine NAG (units/L)	6.4 (2.4, 16.6)	1.8 (1.3, 2.9)	11.9 (7.1, 19)	5.3 (2.7, 9.2) †
Urine β <sub>2</sub> MG (µg/L)	154 (33, 778)	9.5 (0.3, 42)	400 (134, 1118)	171 (64, 1368) †
$E_{Cd}/C_{cr} \times 100$ , µg/L	3.1 (1.1, 7.0)	0.3 (0.3, 0.6)	3.0 (1.7, 5.0)	8.2 (4.8, 15) †
$E_{NAG}/C_{cr} \times 100$ , units/L	5.6 (3.4, 9.4)	3.2 (2.0, 4.1)	8.0 (5.8, 12)	4.9 (3.1, 8.3) †
$E_{\beta_2MG}/C_{cr} \times 100$ , µg/L	137 (29, 604)	17 (0.4, 36)	368 (92, 808)	162 (64, 176) †

SBP = systolic blood pressure; DBP = diastolic blood pressure; MBP = Mean arterial pressure; CKD = chronic kidney disease; eGFR = estimated glomerular filtration rate; NAG = N-acetyl-β-D-glucosaminidase. MBP = DBP + (pulse pressure)/3, where pulse pressure = SBP – DBP. Data for age and eGFR are arithmetic mean values ± standard deviation (SD). Data for blood pressure are geometric mean values ± SD. Data for all other continuous variables are the median (25th, 75th percentile) values. \* Significant % differences among three groups ( $p < 0.05$ , Pearson Chi-Square test). \*\* Significant % differences between two groups ( $p < 0.001$ , Pearson Chi-Square test). † Significant mean differences among three groups ( $p < 0.001$ , Kruskal–Wallis test). ¶ Significant difference from the low exposure group ( $p = 0.003$ , Mann–Whitney U-test). ¶¶ Significant difference from the low exposure group ( $p = 0.014$ , Mann–Whitney U-test).

Mean eGFR fell and the percentages of stages 2 and 3 CKD rose with intensity of exposure. The mean serum creatinine concentration was higher in the high-Cd locality. In general, urine concentrations of Cd, NAG, and β<sub>2</sub>MG rose with Cd exposure, but [NAG]<sub>u</sub> and [β<sub>2</sub>MG]<sub>u</sub> were higher in the moderate Cd group than in the other two.  $E_{Cd}/C_{cr}$ ,  $E_{NAG}/C_{cr}$ , and  $E_{\beta_2MG}/C_{cr}$  followed the same pattern.

Figure 1A–D present scatterplots of eGFR against  $\log(E_{Cd}/C_{cr})$  in each exposure subset and the entire sample. In each subset, significant linear and quadratic relationships were documented, and quadratic  $R^2$  values were slightly higher. Quadratic  $R^2$  was 0.228 in the low-Cd group, 0.083 in the

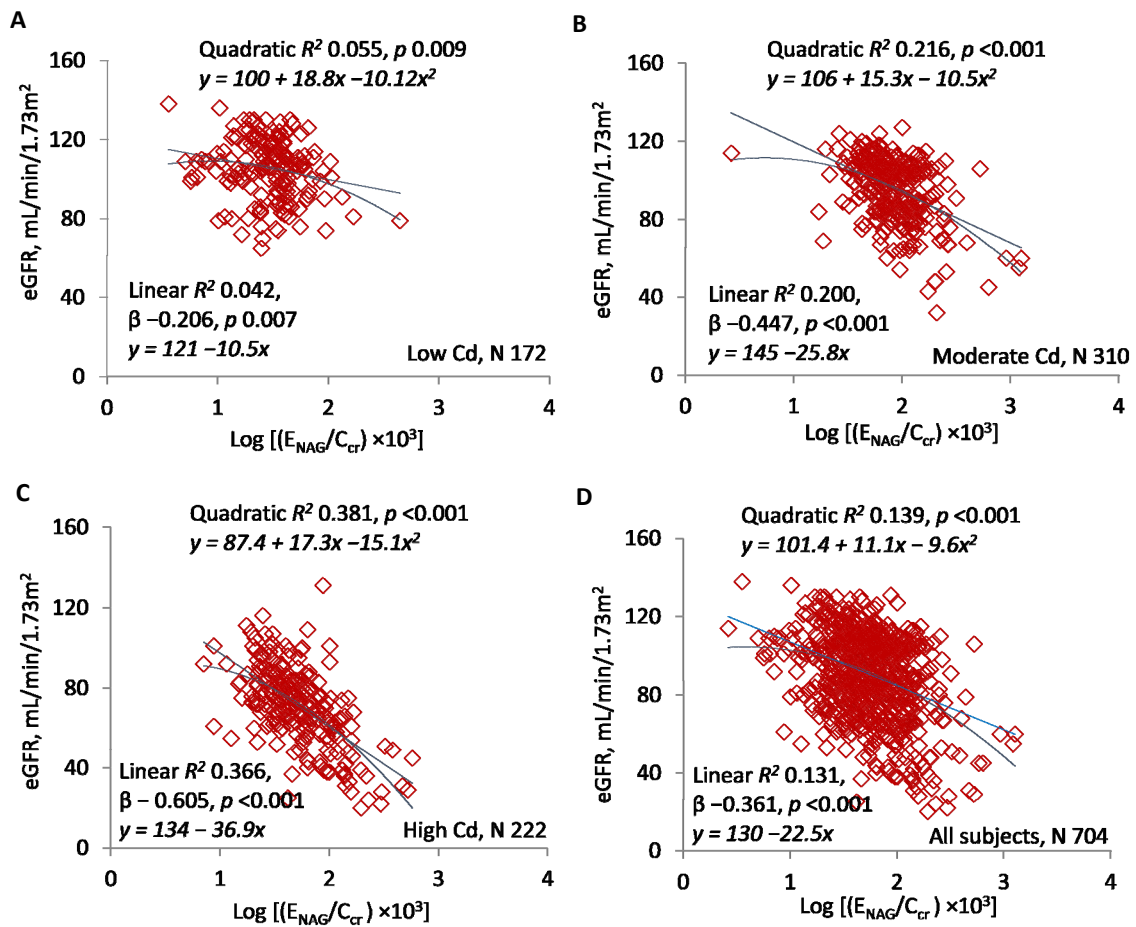
moderate-Cd group, 0.154 in the high-Cd group, and 0.378 in the entire sample. In the linear model, standardized  $\beta$  was  $-0.467$  in the low-Cd group,  $-0.259$  in the moderate-Cd group,  $-0.361$  in the high-Cd group, and  $-0.598$  in the entire sample.



**Figure 1.**  $E_{Cd}/C_{cr}$  as a predictor of the estimated glomerular filtration rate (eGFR). Scatterplots compare eGFR to  $\log[(E_{Cd}/C_{cr}) \times 10^5]$  in subjects grouped by locality (A–C) and in all subjects (D). Quadratic and linear coefficients of determination ( $R^2$ ) are provided together with corresponding equations, standardized  $\beta$  coefficients, and  $p$ -values.

Figure 2A–D present scatterplots of eGFR against  $\log(E_{NAG}/C_{cr})$ . As in Figure 1, significant, inverse linear and quadratic relationships were documented in subsets and the entire sample, and quadratic  $R^2$  values were slightly higher. Quadratic  $R^2$  was 0.055 in the low-Cd group, 0.216 in the moderate-Cd group, 0.381 in the high-Cd group, and 0.139 in the entire sample. In the linear model, standardized  $\beta$  was  $-0.206$  in the low-Cd group,  $-0.447$  in the moderate-Cd group,  $-0.605$  in the high-Cd group, and  $-0.361$  in the entire sample.

The quadratic curves in Figures 1D and 2D indicated that slopes describing rates of GFR reduction varied over the ranges of  $\log[(E_{Cd}/C_{cr}) \times 10^5]$  and  $\log[(E_{NAG}/C_{cr}) \times 10^3]$ . We assumed that  $\log[(E_{Cd}/C_{cr}) \times 10^5]$  of 3.0 and  $\log[(E_{NAG}/C_{cr}) \times 10^3]$  of 1.5 represented the excretion rates of Cd and NAG at which the rates of GFR reduction increased. Table 2 confirms that the slopes changed significantly at these points on the  $x$ -axes.



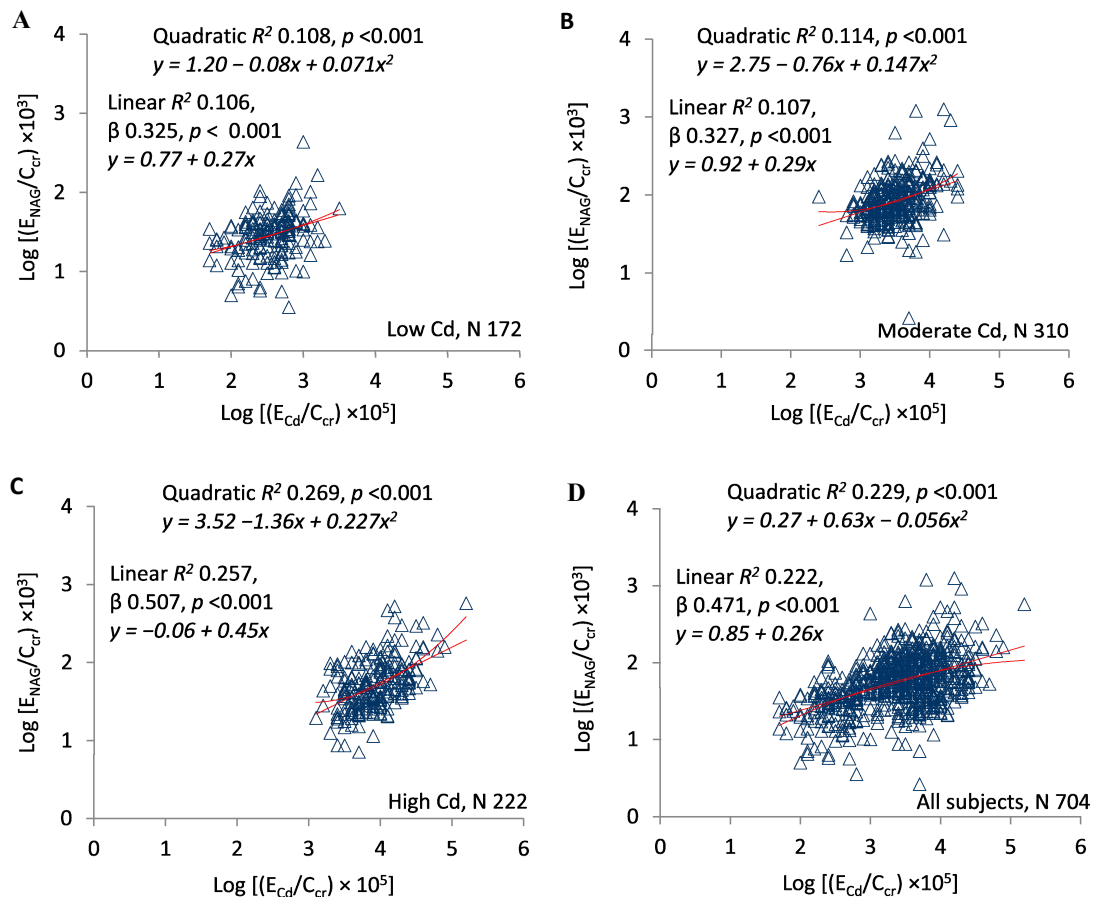
**Figure 2.**  $E_{NAG}/C_{cr}$  as a predictor of eGFR. Scatterplots compare eGFR to  $\log[(E_{NAG}/C_{cr}) \times 10^3]$  in subjects grouped by locality (A–C) and in all subjects (D). Quadratic and linear coefficients of determination ( $R^2$ ) are provided together with corresponding equations, standardized  $\beta$  coefficients, and  $p$ -values.

**Table 2.** Slope analysis for comparing rates of eGFR reduction.

Excretion Rates of Cd or NAG	Number of Subjects	eGFR vs. $\log[(E_{Cd}/C_{cr}) \times 10^5]$ or $\log[(E_{NAG}/C_{cr}) \times 10^3]$				
		$\beta$ Coefficients		$R^2$	$p$ Value	
		Slope (Unstandardized $\beta$ ) $\pm$ SE	Standardized $\beta$			
$\log[(E_{Cd}/C_{cr}) \times 10^5]$	<3	168	$-17.62 \pm 3.20$	-0.392	0.154	<0.001
	$\geq 3$	536	$-27.71 \pm 2.06$	-0.503	0.253	<0.001
All subjects	704		$-20.86 \pm 1.06$	-0.598	0.357	<0.001
$\log[(E_{NAG}/C_{cr}) \times 10^3]$	<1.5	153	$-15.91 \pm 7.62$	-0.168	0.028	0.038
	$\geq 1.5$	551	$-28.35 \pm 3.35$	-0.339	0.115	<0.001
All subjects	704		$-22.49 \pm 2.19$	-0.361	0.131	<0.001

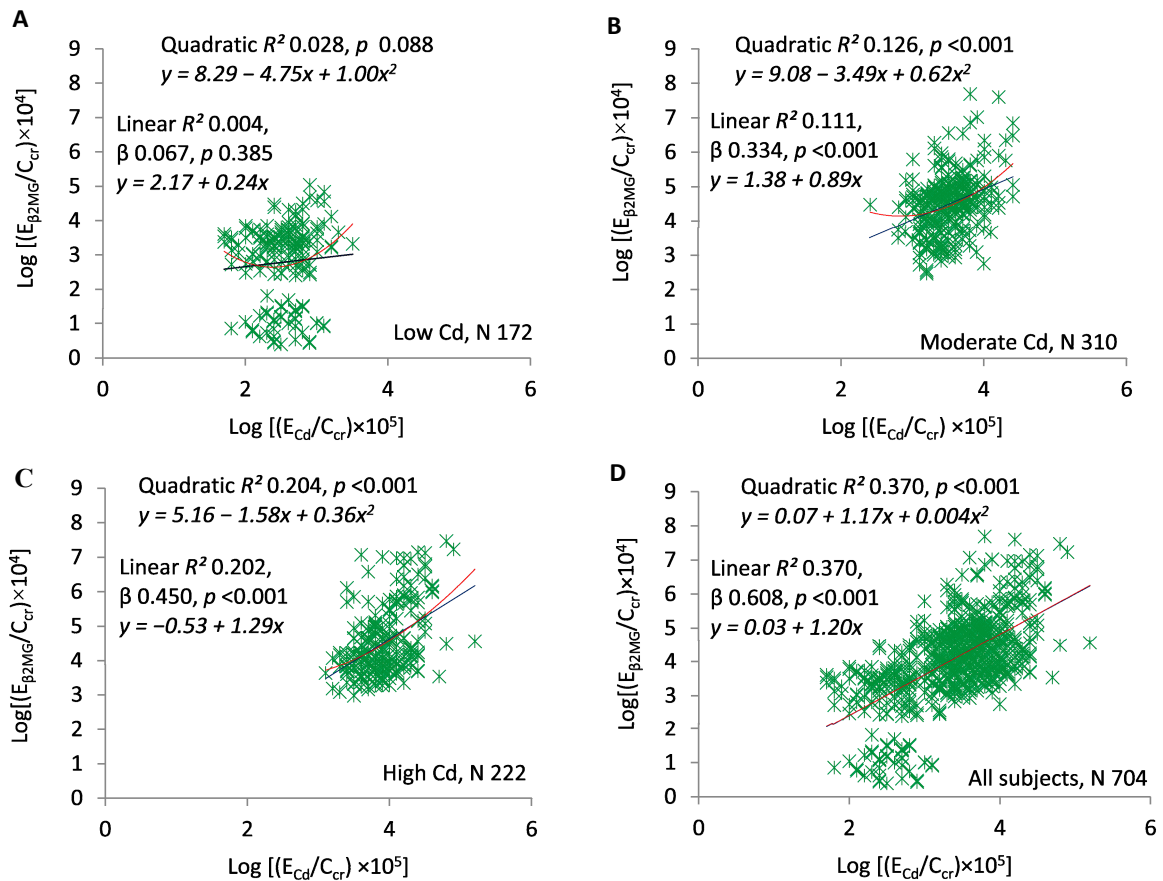
The standardized  $\beta$  coefficient indicates the strength of the association of eGFR with  $\log[(E_{Cd}/C_{cr}) \times 10^5]$  or  $\log[(E_{NAG}/C_{cr}) \times 10^3]$ .  $R^2$  values are coefficients of determination that indicate the fraction of eGFR variation explained by  $E_{Cd}/C_{cr}$  or  $E_{NAG}/C_{cr}$ .  $p \leq 0.05$  identifies statistically significant eGFR reduction rates or associations of eGFR with urinary Cd or NAG excretion.

Figure 3A–D present scatterplots of  $\log(E_{\text{NAG}}/C_{\text{cr}})$  against  $\log(E_{\text{Cd}}/C_{\text{cr}})$  in the exposure subsets and the entire sample. In all subsets, the two ratios varied directly, significant linear and quadratic relationships were documented, and quadratic  $R^2$  values were slightly higher. Quadratic  $R^2$  was 0.108 in the low-Cd group, 0.114 in the moderate-Cd group, 0.269 in the high-Cd group, and 0.229 in the entire sample. In the linear model, standardized  $\beta$  was 0.325 in the low-Cd group, 0.327 in the moderate-Cd group, 0.507 in the high-Cd group, and 0.471 in the entire sample.



**Figure 3.**  $E_{\text{Cd}}/C_{\text{cr}}$  as a predictor of  $E_{\text{NAG}}/C_{\text{cr}}$ . Scatterplots compare  $\log[(E_{\text{NAG}}/C_{\text{cr}}) \times 10^3]$  to  $\log[(E_{\text{Cd}}/C_{\text{cr}}) \times 10^5]$  in subjects grouped by locality (A–C) and in all subjects (D). Quadratic and linear coefficients of determination ( $R^2$ ) are provided together with corresponding equations, standardized  $\beta$  coefficients, and  $p$ -values.

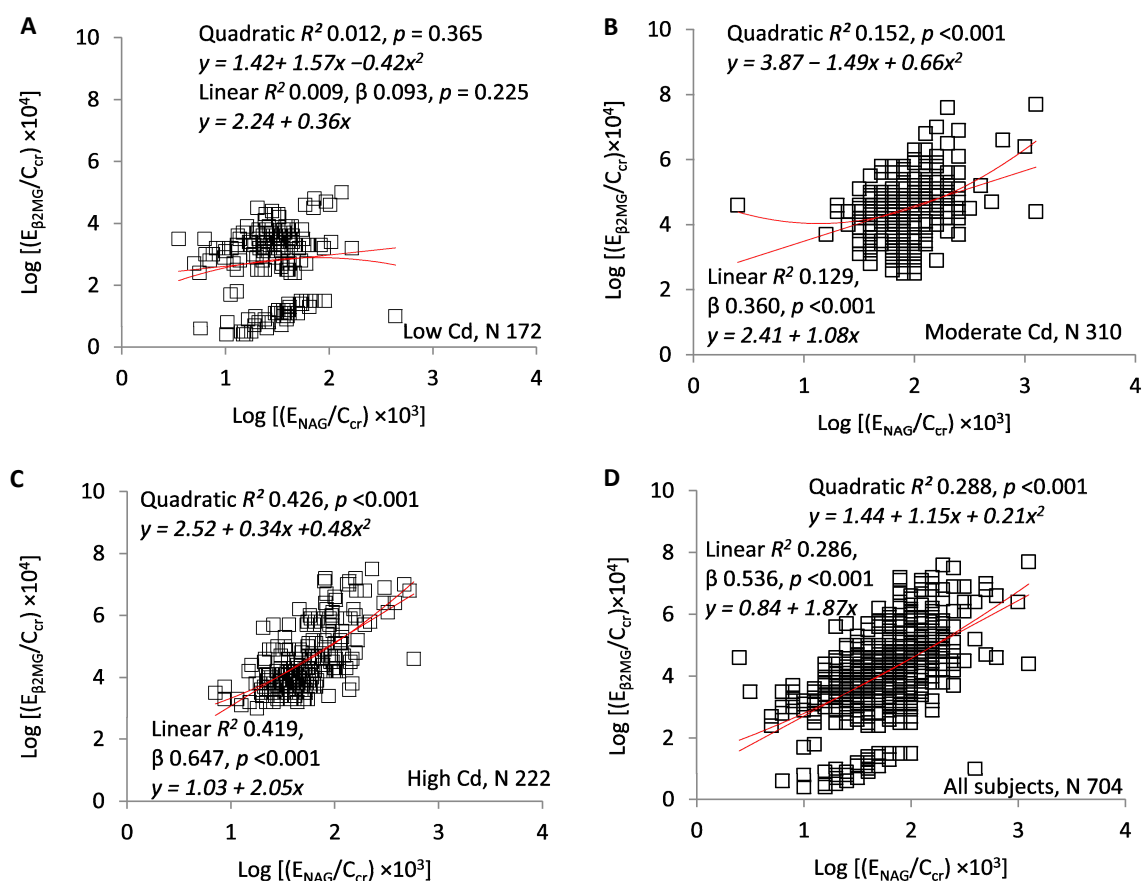
Figure 4A–D present scatterplots of  $\log(E_{\beta 2\text{MG}}/C_{\text{cr}})$  against  $\log(E_{\text{Cd}}/C_{\text{cr}})$  in the exposure subsets and the entire sample. Figure 4A demonstrates the absence of a relationship at the lowest Cd exposure (quadratic  $R^2 = 0.028$ ,  $p = 0.088$ ). At moderate and high exposure, the two ratios were directly related, significant linear and quadratic relationships were documented, and quadratic  $R^2$  values were slightly higher (Figure 4B,C). Quadratic  $R^2$  was 0.126 in the moderate exposure group, 0.204 in the high exposure group, and 0.370 in the entire sample. Quadratic and linear relationships in the entire sample were virtually identical (Figure 4D). In the linear model, standardized  $\beta$  was 0.067 in the low-Cd group, 0.334 in the moderate-Cd group, 0.450 in the high-Cd group, and 0.608 in the entire sample.



**Figure 4.** ECd/C<sub>cr</sub> as a predictor of E<sub>β2MG</sub>/C<sub>cr</sub>. Scatterplots compare log[(E<sub>β2MG</sub>/C<sub>cr</sub>) × 10<sup>4</sup>] to log[(E<sub>Cd</sub>/C<sub>cr</sub>) × 10<sup>5</sup>] in subjects grouped by locality (A–C) and in all subjects (D). Quadratic and linear coefficients of determination (R<sup>2</sup>) are provided together with corresponding equations, standardized β coefficients, and p-values.

Figure 5A–D present scatterplots of log(E<sub>β2MG</sub>/C<sub>cr</sub>) against log(E<sub>NAG</sub>/C<sub>cr</sub>). Figure 5A demonstrates the absence of a relationship at the lowest Cd exposure (linear R<sup>2</sup> = 0.009, p = 0.225). At moderate and high exposure, the two ratios were directly related, significant linear and quadratic regressions were documented, and quadratic R<sup>2</sup> values were slightly higher (Figure 5B,C). Quadratic R<sup>2</sup> was 0.152 in the moderate exposure group, 0.426 in the high exposure group, and 0.288 in the entire sample. In the linear model, standardized β was 0.093 in the low-Cd group, 0.360 in the moderate-Cd group, 0.647 in the high-Cd group, and 0.536 in the entire sample.





**Figure 5.**  $E_{NAG}/C_{cr}$  as a predictor of  $E_{\beta 2MG}/C_{cr}$ . Scatterplots compare  $\log[(E_{NAG}/C_{cr}) \times 10^3]$  to  $\log[(E_{\beta 2MG}/C_{cr}) \times 10^4]$  in subjects grouped by locality (A–C) and in all subjects (D). Quadratic and linear coefficients of determination ( $R^2$ ) are provided together with corresponding equations, standardized  $\beta$  coefficients, and  $p$ -values.

#### 4. Discussion

Our goals in the present study were to elucidate the source of urinary Cd and to relate that source to the pathogenesis of Cd nephropathy. Data were obtained from clinically healthy Thai subjects residing in areas with low, moderate, or high exposure to Cd. In Mae Sot District, Tak Province, intensity of exposure was determined from the Cd content of rice grains [35,36]. Exposure in Bangkok was assumed to be low on the basis of food analyses and dietary histories [33].

Subjects in the three subsets were demographically dissimilar (Table 1). Both age and percentage of smokers rose with intensity of exposure. The percentage of women was particularly high in the moderate exposure group, and some women were of childbearing age. Because iron and Cd share a transporter in intestinal epithelium, menstruating women in this group may have absorbed Cd with exceptional avidity and incurred exceptional tubular toxicity secondarily (Table 1). In the high exposure subset, increased age may have conferred additional reasons for deterioration of GFR, and smoking may have provided a second environmental source of Cd. Whether smoking itself could have accelerated the progression of CKD is unresolved [40–42]. Neither age, nor smoking per se, nor the source of exogenous Cd obscured the significant relationship between eGFR and  $E_{Cd}/C_{cr}$ .

Table 1 shows that with the increasing intensity of exposure,  $E_{Cd}/C_{cr}$  rose and eGFR fell in stepwise fashion. In contrast, both  $E_{NAG}/C_{cr}$  and  $E_{\beta 2MG}/C_{cr}$  were higher in the moderate than in the high exposure group. Although one might expect a direct relationship between NAG excretion and the number of intact nephrons, the higher  $E_{NAG}/C_{cr}$  in the moderate group implies that the median excretion of NAG *per intact nephron* was also higher in these subjects. At the same time, the overlap of  $E_{NAG}/C_{cr}$  between the moderate and high exposure groups was substantial (Figures 2 and 5), and

consistent relationships among  $E_{Cd}/C_{cr}$ ,  $E_{NAG}/C_{cr}$ , and eGFR were demonstrable at all intensities of exposure (Figures 1–3). We speculate that the number of menstruating women in the moderate exposure subset was sufficient to increase Cd absorption and tubular toxicity in the entire group, but insufficient to disrupt the statistical relationships seen in all groups among  $E_{Cd}/C_{cr}$ ,  $E_{NAG}/C_{cr}$ , and eGFR.

Analogous statements can be made about  $\beta_2MG$ . As impaired reabsorption of this protein is an early sign of proximal tubular injury, it is not surprising that median  $E_{\beta_2MG}/C_{cr}$  tracked with median  $E_{NAG}/C_{cr}$  (Table 1). As would be expected,  $E_{\beta_2MG}/C_{cr}$  varied directly with  $E_{NAG}/C_{cr}$  in the moderate and high exposure groups, but also varied directly with  $E_{Cd}/C_{cr}$ , which increased progressively with the intensity of exposure (Figures 4 and 5).

Although CKD-EPI equations estimate GFR imprecisely [37,38], each exposure group differed significantly from the others with respect to eGFR (Table 1). Estimated GFR was inversely related to  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$  in the entire sample and each subset (Figures 1 and 2), and  $E_{NAG}/C_{cr}$  varied directly with  $E_{Cd}/C_{cr}$  (Figure 3). For all comparisons in Figures 1–3, both linear and quadratic relationships were significant, and with one exception (Figure 1B),  $R^2$  rose with the exposure intensity. Standardized  $\beta$  followed the same pattern. Despite the statistical significance of all comparisons, some  $R^2$  values indicated that the fractional contribution of  $E_{Cd}/C_{cr}$  or  $E_{NAG}/C_{cr}$  to eGFR was <10% (Figures 1B and 2A); simultaneously, however, standardized  $\beta$  indicated robust effects of changes in  $x$  on changes in  $y$ . Factors other than  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$  affected eGFR, but variation in each ratio was associated with substantial variation in eGFR.

Taken together, the graphs in Figures 1–3 imply that in each subset, GFR was inversely related to the severity of cellular injury per nephron ( $E_{NAG}/C_{cr}$ ), which in turn was associated with the amount of Cd excreted per nephron ( $E_{Cd}/C_{cr}$ ). In addition, the quadratic relationships in Figures 1 and 2 suggest that small increments in the most advanced injury were accompanied by disproportionate reductions in GFR. Slope analyses of curves in Figures 1D and 2D confirm this inference (Table 2).

Other investigators have described direct relationships of  $[NAG]_u/[cr]_u$  and eGFR to  $[Cd]_u/[cr]_u$ , but we have not found a synthesis of those relationships into a satisfactory pathophysiologic narrative [35,43–48]. A cogent interpretation of Figures 1–3 must explain how eGFR and  $E_{NAG}/C_{cr}$ —results of cumulative Cd sequestration—were related physiologically to  $E_{Cd}/C_{cr}$ , an indicator of Cd excretion at the time of sampling. Cd was excreted for two possible reasons; it was filtered and not reabsorbed, or it was released from tubular cells [32]. Although both processes may have occurred, we do not see how the first, excretion after filtration, could have produced a physiologic connection between  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$  (Figure 3). In contrast, if Cd was released from damaged tubules, then Cd and NAG emanated from the same source, and both  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$  measured cellular injury. This shared attribute of Cd and NAG explains the statistical association of the ratios.

Additional evidence for the tubular origin of excreted Cd is provided by demonstrated extrusions of MT into tubular lumens [49], documented correlations of  $E_{Cd}$  with renal tissue content of Cd [50–52], and direct relationships between  $E_{Cd}$  and GFR (number of intact nephrons) [53–55]. Experiments in rabbits demonstrated a high tubular maximum for reabsorption of CdMT that would preclude excretion of filtered Cd in the typically intoxicated human [56].

In addition to addressing the likely source of excreted Cd, we must also ask why declining eGFR, the result of continuous loss of intact nephrons over time, was associated in the present study with parameters of current cellular injury,  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$ . To address this paradox, we propose that eGFR,  $E_{Cd}/C_{cr}$ , and  $E_{NAG}/C_{cr}$  were simultaneous consequences of the tubular content of Cd. As the content rose, cellular injury per nephron and the rate of nephron loss increased proportionately; at any moment in a subject's exposure history, the three variables were quantitatively associated because they were traceable to the same burden of sequestered Cd.

$\beta_2MG$ , a small protein made by nucleated cells, is almost completely filtered by glomeruli [29]. Ordinarily, the proximal tubule reabsorbs and degrades over 99% of filtered  $\beta_2MG$  [30]. Because tubulopathies increase  $E_{\beta_2MG}$  [31], excessive  $E_{\beta_2MG}$  is conventionally interpreted as evidence of

reabsorptive dysfunction [35,57,58]. This interpretation is understandable, but we suspect that it is an oversimplification. One reason is that endogenous production of  $\beta_2$ MG may be increased by chronic inflammatory conditions, solid tumors, lymphatic malignancies, and multiple myeloma [59]. If tubular degradation ( $TD_{\beta_2MG}$ ) remains constant as production rises,  $E_{\beta_2MG}$  also rises even though  $TD_{\beta_2MG}$  has not fallen (SM). Moreover, if both  $\beta_2$ MG production and  $TD_{\beta_2MG}$  per volume of filtrate ( $TD_{\beta_2MG}/C_{cr}$ ) remain constant as GFR falls,  $TD_{\beta_2MG}$  also falls, and  $E_{\beta_2MG}$  rises (SM). Although these inferences are unproven, it seems likely that a combination of reabsorptive dysfunction and reduced GFR caused associations of  $E_{\beta_2MG}/C_{cr}$  with  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$  (Figures 4 and 5).

An additional observation requires explanation. In the low exposure group, a cluster of subjects exhibited exceptionally low  $E_{\beta_2MG}/C_{cr}$ . At a fixed rate of  $\beta_2$ MG filtration (equal to endogenous production) and a fixed value of  $TD_{\beta_2MG}/C_{cr}$ , the rate of  $\beta_2$ MG reabsorption increases with the number of intact nephrons, and  $E_{\beta_2MG}$  decreases simultaneously. In the isolated cluster, mean eGFR was 105.3 mL/min/1.73 m<sup>2</sup>; in the remainder of subjects in the study, it was 89.3 mL/min/1.73 m<sup>2</sup>. We suspect that extremely low  $E_{\beta_2MG}/C_{cr}$  in the cluster was the result of high  $C_{cr}$ , high  $TD_{\beta_2MG}$ , and secondarily reduced  $E_{\beta_2MG}$ .

In the present study, we have continued the recently introduced practice of normalizing excretion of Cd, NAG, and  $\beta_2$ MG to creatinine clearance instead of creatinine excretion [26]. Because the resulting ratios express  $E_{Cd}$ ,  $E_{NAG}$ , and  $E_{\beta_2MG}$  as functions of intact nephron mass, they nullify sources of imprecision that accompany normalization to  $E_{cr}$  or  $[cr]_u$ . At any GFR,  $E_{cr}$  is primarily a function of muscle mass [60]; consequently, at a given  $E_{Cd}$  (for example),  $[Cd]_u/[cr]_u$  may vary by a multiple over the range of human body size. Moreover, multiple groups have reported *direct* rather than inverse relationships between GFR and  $[Cd]_u/[cr]_u$  after Cd exposure [5,53–55]. If the nephron number determines  $E_{Cd}$  at a given cellular burden of the metal, then  $[Cd]_u/[cr]_u$  may exaggerate the burden at normal GFR and underestimate it at reduced GFR. Normalization of  $E_{Cd}$  to  $C_{cr}$ —that is, calculation of  $[Cd]_u[cr]_p/[cr]_u$ —eliminates the confounding effects of both muscle mass and nephron number on  $[Cd]_u/[cr]_u$ . In addition, because the required measurements are made in aliquots of urine and serum, the calculation quantifies amounts of Cd (or other substances) excreted per volume of filtrate while eliminating the need for timed urine collections and direct determinations of GFR. We plan to address optimal expression of excretion rates relevant to Cd nephropathy in a separate publication.

In summary, we draw the following conclusions from the significant regressions described herein.  $E_{NAG}/C_{cr}$  varied directly with  $E_{Cd}/C_{cr}$  because sequestered Cd induced the release of NAG and Cd from tubular cells into filtrate. Estimated GFR varied inversely with both ratios because all three parameters reflected the extent of tubular Cd accumulation.  $E_{NAG}/C_{cr}$  and  $E_{Cd}/C_{cr}$  quantified ongoing cellular injury, and eGFR quantified the loss of intact nephrons. We suspect that the significant regressions of  $E_{\beta_2MG}/C_{cr}$  on  $E_{Cd}/C_{cr}$  and  $E_{NAG}/C_{cr}$  resulted from effects of Cd on both tubular reabsorption and nephron number.

**Supplementary Materials:** The following are available online at <http://www.mdpi.com/2305-6304/7/4/55/s1>, The equation for normalization to creatinine clearance.

**Author Contributions:** S.S., D.A.V., W.R., M.N., and G.C.G. formulated the study designs and protocols. S.S. and W.R. obtained ethical institutional clearances for research on human subjects and supervised the collection of biologic specimens in Thailand. S.S. organized and analyzed the data, created the tables and figures, and revised the manuscript for important intellectual content. K.R.P. proposed normalization of excretion rates to creatinine clearance, provided logical data interpretation, and was the primary author of the manuscript.

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## Abbreviations

Abbreviation	Meaning
Cd	Cadmium
GFR	Glomerular filtration rate, units of volume/time
eGFR	Estimated glomerular filtration rate, units of mL/min/1.73 m <sup>2</sup>
CKD-EPI	Chronic kidney disease epidemiology collaboration
MT	Metallothionein
CdMT	Cadmium–metallothionein complex
PC	Phytochelatin
CdPC	Cadmium–phytochelatin complex
GSH	Glutathione
NAG	N-acetyl- $\beta$ -D-glucosaminidase
$\beta_2$ MG	Beta <sub>2</sub> -microglobulin
C <sub>cr</sub>	Creatinine clearance, units of volume/time
V <sub>u</sub>	Urine flow rate, units of volume/time
E <sub>x</sub> /C <sub>cr</sub>	Excretion rate of x per volume of filtrate, units of mass/volume, where x = Cd, NAG, or $\beta_2$ MG
TD <sub><math>\beta_2</math>MG</sub>	Rate of tubular degradation of $\beta_2$ MG, units of mass/time
TD <sub><math>\beta_2</math>MG</sub> /C <sub>cr</sub>	Amount of $\beta_2$ MG degraded per volume of filtrate, units of mass/volume

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