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The Effect of Cadmium on GFR Is Clarified by Normalization of Excretion Rates to Creatinine Clearance

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Abstract: Erroneous conclusions may result from normalization of urine cadmium and N-acetyl-β-D-glucosaminidase concentrations ($[Cd]_u$ and $[NAG]_u$) to the urine creatinine concentration ($[cr]_u$). In theory, the sources of these errors are nullified by normalization of excretion rates (E_{Cd} and E_{NAG}) to creatinine clearance (C_{cr}). We hypothesized that this alternate approach would clarify the contribution of Cd-induced tubular injury to nephron loss. We studied 931 Thai subjects with a wide range of environmental Cd exposure. For x = Cd or NAG, E_x/E_{cr} and E_x/C_{cr} were calculated as $[x]_u/[cr]_u$ and $[x]_u[cr]_p/[cr]_u$, respectively. Glomerular filtration rate (GFR) was estimated according to the Chronic Kidney Disease (CKD) Epidemiology Collaboration (eGFR), and CKD was defined as eGFR < 60 mL/min/1.73m^2. In multivariable logistic regression analyses, prevalence odds ratios (PORs) for CKD were higher for $\log(E_{Cd}/C_{cr})$ and $\log(E_{NAG}/C_{cr})$ than for $\log(E_{Cd}/E_{cr})$ and $\log(E_{NAG}/E_{cr})$. Doubling of E_{Cd}/C_{cr} and E_{NAG}/C_{cr} increased POR by 132% and 168%; doubling of E_{Cd}/E_{cr} and E_{NAG}/E_{cr} increased POR by 64% and 54%. As $\log(E_{Cd}/C_{cr})$ rose, associations of eGFR with $\log(E_{Cd}/C_{cr})$ and $\log(E_{NAG}/C_{cr})$ became stronger, while associations of eGFR with $\log(E_{Cd}/E_{cr})$ and $\log(E_{NAG}/E_{cr})$ became insignificant. In univariate regressions of eGFR on each of these logarithmic variables, R^2 was consistently higher with normalization to C_{cr}. Our tabular and graphic analyses uniformly indicate that normalization to C_{cr} clarified relationships of E_{Cd} and E_{NAG} to eGFR.

Keywords: cadmium; chronic kidney disease; excretion rate; GFR; N-acetyl-β-D-glucosaminidase; nephrotoxicity; urine creatinine

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1. Introduction

Cadmium (Cd) is an important industrial toxin in several regions of the world [1]. It enters the human body in food, cigarette smoke, and polluted air, and is initially bound to the protein metallothionein (MT) in the liver. Complexes of CdMT are subsequently released to the circulation, filtered by renal glomeruli, and reabsorbed by proximal tubules. After Cd is separated from MT in lysosomes of tubular cells, it induces in situ synthesis of MT, which mitigates the toxicity of the free metal. Nevertheless, a small amount of unbound Cd inflicts injury that may eventuate in nephron loss and a reduction in the glomerular filtration rate (GFR) [2,3].

The lysosomal enzyme N-acetyl-β-D-glucosaminidase (NAG) is the most commonly employed marker of Cd-induced cell injury. Because this molecule is too large to undergo glomerular filtration, its appearance in urine signifies release by injured tubular cells [4]. The excretion rate of NAG (E_{NAG}) typically correlates with that of Cd (E_{Cd}) [5–11], and the correlation holds at minimal Cd excretion rates [12–14]. Given these observations, we have argued that the two substances probably emanate from the same source [15]. We recently showed that E_{Cd} and E_{NAG} were inversely related to estimated glomerular filtration rate (eGFR) in a sample of Thai subjects [15].

Historically, investigators of Cd nephrotoxicity have normalized the excretion rates of various substances (E_x) to that of creatinine (E_{cr}) [7,9–12,16,17]. Algebraically, E_x/E_{cr} simplifies to $[x]_u/[cr]_u$. Although normalization of $[x]_u$ to $[cr]_u$ corrects for the effect of urine flow rate (V_u) on $[x]_u$, this convention introduces two other sources of error. First, because E_{cr} is primarily a function of muscle mass [18], $[x]_u/[cr]_u$ may vary by a multiple among subjects with a given E_x. Second, if Cd and NAG emanate from tubular cells, their excretion rates may fall as nephrons are lost while E_{cr} remains relatively stable [19–21]. In this circumstance, E_{Cd}/E_{cr} and E_{NAG}/E_{cr} may understate the severity of Cd-induced injury.

To avoid these potential errors, we recently adopted the practice of normalizing E_x to creatinine clearance (C_{cr}), a surrogate for the glomerular filtration rate (GFR) [15,22]. C_{cr} is the excretion rate divided by the plasma concentration of creatinine ($E_{cr}/[cr]_p$); algebraically, E_x/C_{cr} simplifies to $[x]_u[cr]_p/[cr]_u$ in units of mass of x excreted per volume of filtrate [23]. Because the ratio $[x]_u/[cr]_u$ remains in the calculation, E_x/C_{cr}, like E_x/E_{cr}, is unaffected by V_u. However, in contrast to E_x/E_{cr}, E_x/C_{cr} is also unaffected by muscle mass because $[cr]_p$ varies in proportion to E_{cr} at a given C_{cr}. Moreover, if x emanates from tubular cells, E_x may fall because of nephron loss; in that circumstance, E_x/C_{cr} depicts the excretion of x per surviving nephron because C_{cr} falls simultaneously [2,3].

Given the foregoing considerations, we hypothesized that normalization of E_{Cd} and E_{NAG} to C_{cr} would clarify the contribution of Cd-induced tubular injury to nephron loss. We employed data from a large sample of Thai subjects to examine regressions of estimated GFR (eGFR) on $\log(E_{Cd}/E_{cr})$, $\log(E_{NAG}/E_{cr})$, $\log(E_{Cd}/C_{cr})$, and $\log(E_{NAG}/C_{cr})$. Normalization to C_{cr} increased coefficients of determination, effect sizes, and the strength of associations of eGFR with excretion rates.

2. Results

2.1. Tabular Analyses

Table 1 summarizes demographic features, renal function, and excretion rates of Cd and NAG in a cohort of 931 subjects. Data were organized in three subsets defined by gradations of $\log[(E_{Cd}/C_{cr}) \times 10^5]$. As $\log(E_{Cd}/C_{cr})$ rose, age, $[Cd]_u$, $[NAG]_u$, E_{Cd}/E_{cr}, E_{NAG}/E_{cr}, E_{Cd}/C_{cr}, and E_{NAG}/C_{cr} also rose, and eGFR fell.

Table 2 presents two logistic regression models that quantified contributions of independent variables to the prevalence odds ratio (POR) for CKD (eGFR < 60 mL/min/1.73m^2). Both models incorporated demographic factors and parameters of Cd and NAG excretion. E_{Cd} and E_{NAG} were normalized to E_{cr} in model 1 and to C_{cr} in model 2.

In model 1, Table 2, POR for CKD was associated with age, $\log_2(E_{Cd}/E_{cr})$, and $\log_2(E_{NAG}/E_{cr})$, but not with diabetes, gender, hypertension, or smoking. $\text{Log}_2(E_{Cd}/E_{cr})$ had the greatest effect size (β = 0.493), followed by $\log_2(E_{NAG}/E_{cr})$ (β = 0.435) and age (β = 0.143). In model 2, results were qualitatively similar; POR was associated with age, $\log_2(E_{Cd}/C_{cr})$, and $\log_2(E_{NAG}/C_{cr})$, but not with diabetes, gender, hypertension, or smoking. In contrast to model 1, $\log_2(E_{NAG}/C_{cr}$ had the greatest effect size (β = 0.985), followed by $\log_2 E_{Cd}/C_{cr}$ (β = 0.843) and age (β = 0.149). Effect size on POR was much greater for $\log_2(E_{Cd}/C_{cr})$ and $\log_2(E_{NAG}/C_{cr})$ than for $\log_2(E_{Cd}/E_{cr})$ and $\log_2(E_{NAG}/E_{cr})$. Doubling of E_{Cd}/E_{cr} and E_{NAG}/E_{cr} increased POR for CKD by 63.7% and 54.4%, respectively; doubling of E_{Cd}/C_{cr} and E_{NAG}/C_{cr} increased POR by 132% and 168%, respectively. In both models, POR for CKD rose by 14–15% with every 10-year increment above age 40.

Table 1. Study subjects grouped by urinary cadmium excretion rates normalized to creatinine clearance.

Parameters/Factors	All Subjects	$Log[(E_{Cd}/C_{cr}) \times 10^5]$		
	***n* = 931**	**<2.5, *n* = 146**	**2.5−3.9, *n* = 654**	**≥4.0, *n* = 131**
Age (years)	44.4 ± 12.5	31.9 ± 9.1	44.8 ±10.9	56.3 ± 10.8 *
Age range	16−87	16−53	18−87	36−83
eGFR (mL/min/1.73 m^2) [a]	93.8 ± 20.8	109.3 ± 12.0	95.3 ± 17.7	69.1 ± 21.8 *
eGFR range	20−139	78−130	25−139	20−112
eGFR <60 mL/min/1.73 m^2 (%)	7.1	0	3.5	32.8 †
Women (%)	58.5	54.1	60.4	54.2
Smoking (%)	39.2	19.2	29.8	58.8 †
Hypertension (%)	25.5	8.2	28.8	26.0 †
Diabetes mellitus (%)	1.2	0	0.6	5.3 †
Serum creatinine, mg/dL	0.85 ± 0.26	0.80 ± 0.15	0.82 ± 0.20	1.08 ± 0.42 *
Urine creatinine, mg/dL	81.1 ± 73.7	53.9 ± 63.0	86.7 ± 76.0	91.2 ± 63.9 *
Urine Cd, μg/L	1.52 ± 8.32	0.11 ± 0.17	1.77 ± 3.98	13.57 ± 14.62 *
Urine NAG, units/L	5.48 ± 9.95	1.73 ± 1.67	6.14 ± 10.70	7.19 ± 7.00 *
Normalized to E_{cr} as E_x/E_{cr} [b]				
E_{Cd}/E_{cr}, μg/g creatinine	1.85 ± 6.23	0.19 ± 0.10	2.04 ± 2.81	14.88 ± 8.05 *
E_{NAG}/E_{cr}, units/g creatinine	6.40 ± 8.94	3.27 ± 2.39	6.74 ± 7.63	7.88 ± 13.97 *
Normalized to C_{cr} as E_x/C_{cr} [c]				
$E_{Cd}/C_{cr} \times 100$, μg/L	1.57 ± 7.23	0.15 ± 0.07	1.67 ± 2.24	16.01 ± 11.05 *
$E_{NAG}/C_{cr} \times 100$, units/L	5.42 ± 9.39	2.47 ± 1.93	5.47 ± 7.62	8.48 ± 15.12 *

n = number of subjects; [a] eGFR = estimated glomerular filtration rate, determined with Chronic Kidney Disease Epidemiology Collaboration (CKD−EPI) equations [24]; [b] $E_x/E_{cr} = [x]_u/[cr]_u$; [c] $E_x/C_{cr} = [x]_u[cr]_p/[cr]_u$, where *x* = Cd or NAG [23]. Data for age and eGFR are arithmetic means ± standard deviation (SD). Data for all other continuous variables are geometric means ± SD. Data for urine NAG are from 736 subjects; data for all other variables are from 931 subjects. † Significant % differences among three groups ($p < 0.001$, Pearson Chi-Square test). * Significant mean differences among three groups ($p < 0.001$, Kruskal–Wallis test).

Table 2. Relationships of prevalence odds ratios for chronic kidney disease to demographic factors and parameters of Cd and NAG excretion.

Independent Variables/Factors	eGFR Levels <60 mL/min/1.73 m^2				
	β Coefficients	POR	95% CI		*p*
	(SE)		Lower	Upper	Value
Model 1, n = 736					
Age (years)	0.143 (0.018)	1.153	1.113	1.195	<0.001
$Log_2[(E_{Cd}/E_{cr}) \times 10^3]$, μg/g creatinine	0.493 (0.137)	1.637	1.252	2.141	<0.001
$Log_2[(E_{NAG}/E_{cr}) \times 10^3]$, units/g creatinine	0.435 (0.172)	1.544	1.103	2.163	0.011
Diabetes	0.705 (0.884)	2.023	0.358	11.434	0.425
Gender (women)	−0.014 (0.374)	0.986	0.474	2.054	0.971
Hypertension	0.644 (0.341)	1.903	0.976	3.711	0.059
Smoking	−0.211(0.372)	0.810	0.391	1.679	0.571
Adjusted R^2	0.499	–	–	–	<0.001
Model 2, n = 736					
Age (years)	0.149 (0.021)	1.160	1.113	1.210	<0.001
$Log_2[(E_{Cd}/C_{cr}) \times 10^5]$, μg/L	0.843 (0.161)	2.324	1.695	3.187	<0.001
$Log_2[(E_{NAG}/C_{cr}) \times 10^4]$, units/L	0.985 (0.192)	2.678	1.837	3.905	<0.001
Diabetes	0.100 (0.961)	1.105	0.168	7.264	0.917
Gender (women)	0.459 (0.429)	1.582	0.682	3.669	0.285
Hypertension	0.667 (0.395)	1.949	0.898	4.229	0.091
Smoking	−0.374 (0.414)	0.688	0.305	1.549	0.366
Adjusted R^2	0.544	–	–	–	<0.001

POR = Prevalence Odds Ratio; S.E. = Standard error of mean; Data were generated from logistic regression analyses relating POR for CKD to independent variables. Independent variables are listed in the first column. CKD was defined as eGFR <60 mL/min/1.73m^2. *p*-Values < 0.05 indicate a statistically significant increase in POR for CKD. $Log_2[(E_{Cd}/E_{cr}) \times 10^3]$ and $log_2[(E_{NAG}/E_{cr}) \times 10^3]$ were incorporated into model 1; $log_2[(E_{Cd}/C_{cr}) \times 10^5]$ and $log_2[(E_{NAG}/C_{cr}) \times 10^4]$ were incorporated into model 2. Other independent variables in models 1 and 2 were identical. β coefficients indicate the size of the effect of an independent variable on POR for CKD. Adjusted R^2 values were obtained by univariate analyses that incorporated eGFR as a continuous variable. Independent variables were identical to those used in the logistic regression analyses.

Table 3 presents two multivariable linear regression models of eGFR. As in Table 2, the models incorporated demographic factors and parameters of Cd and NAG excretion. E_{Cd} and E_{NAG} were normalized to E_{cr} in model 1 and to C_{cr} in model 2. Accordingly, subsets of subjects were defined by gradations of $\log[(E_{Cd}/E_{cr}) \times 10^3]$ in model 1 and $\log[(E_{Cd}/C_{cr}) \times 10^5]$ in model 2. In each model, age exhibited the greatest absolute value of standardized β (strength of association) at all three gradations of $\log[(E_{Cd}/E_{cr}) \times 10^3]$ or $\log[(E_{Cd}/C_{cr}) \times 10^5]$.

Table 3. Relationships of eGFR to demographic factors and parameters of Cd and NAG excretion.

Independent Variables/Factors	eGFR, mL/min/1.73 m^2					
	Cd Excretion Level 1		Cd Excretion Level 2		Cd Excretion Level 3	
	β	*p*	β	*p*	β	*p*
Model 1, n = 736						
Age (years)	−0.527	<0.001	−0.624	<0.001	−0.489	<0.001
$Log_{10}[(E_{Cd}/E_{cr}) \times 10^3]$,μg/g creatinine	−0.077	0.422	−0.088	0.023	−0.138	0.061
$Log_{10}[(E_{NAG}/E_{cr}) \times 10^3]$, unit/g creatinine	0.368	<0.001	0.068	0.060	0.056	0.456
Gender (women)	−0.261	0.026	−0.053	0.166	−0.030	0.715
Smoking	−0.256	0.025	0.029	0.455	−0.069	0.392
Hypertension	0.266	0.013	0.049	0.139	0.127	0.091
Diabetes	–	–	0.041	0.201	0.135	0.069
Adjusted R^2	0.531	<0.001	0.454	<0.001	0.290	<0.001
Model 2, n = 736						
Age (years)	−0.640	<0.001	−0.548	<0.001	−0.483	0.001
$Log_{10}[(E_{Cd}/C_{cr}) \times 10^5]$, μg/L	−0.073	0.414	−0.128	0.001	−0.281	0.001
$Log_{10}[(E_{NAG}/C_{cr}) \times 10^4]$, units/L	0.175	0.049	−0.095	0.010	−0.228	0.002
Gender (women)	−0.276	0.010	−0.091	0.023	0.068	0.366
Smoking	−0.109	0.276	−0.040	0.335	0.002	0.980
Hypertension	0.168	0.075	0.029	0.420	0.108	0.125
Diabetes	–	–	0.053	0.125	0.026	0.706
Adjusted R^2	0.452	<0.001	0.383	<0.001	0.436	<0.001

Data were derived from two linear regression models relating eGFR, a continuous dependent variable, to seven independent variables (first column). We incorporated $\log_{10}[(E_{Cd}/E_{cr}) \times 10^3]$ and $\log_{10}[(E_{NAG}/E_{cr}) \times 10^3]$ into model 1 and $\log_{10}[(E_{Cd}/C_{cr}) \times 10^5]$ and $\log_{10}[(E_{NAG}/C_{cr}) \times 10^4]$ into model 2. Units of E_{Cd}/E_{cr} and E_{NAG}/E_{cr} are μg/g cr and units/g cr, respectively. Units of E_{Cd}/C_{cr} and E_{NAG}/C_{cr} are μg/L of filtrate and units/L of filtrate, respectively. Cd excretion level 1 implies $\log_{10}[(E_{Cd}/E_{cr}) \times 10^3]$ and $\log_{10}[(E_{Cd}/C_{cr}) \times 10^5] < 2.5$. Cd excretion level 2 implies $\log_{10}[(E_{Cd}/E_{cr}) \times 10^3]$ and $\log_{10}[(E_{Cd}/C_{cr}) \times 10^5] = 2.5$–3.9. Cd excretion level 3 implies $\log_{10}[(E_{Cd}/E_{cr}) \times 10^3]$ and $\log_{10}[(E_{Cd}/C_{cr}) \times 10^5] \geq 4.0$. Standardized regression coefficients (β) indicate strength of associations between eGFR and independent variables. $p \leq 0.05$ identifies statistical significance. Adjusted R^2 values indicate the total variation in eGFR that was explained by all independent variables incorporated into each model. For model 1, the values for geometric mean (GM) (SD) of E_{Cd}/E_{cr} within Cd excretion levels 1, 2 and 3 were 0.15 (0.07), 2.35 (2.42) and 14.9 (7.57) μg/g creatinine. The corresponding numbers of subjects in the three Cd excretion levels were 57, 534 and 145, respectively. For model 2, the values for GM (SD) of $[E_{Cd}/C_{cr} \times 100]$ (SD) within Cd excretion levels 1, 2 and 3 were 0.15 (0.08), 2.20 (2.25) and 16.01 (11.05) μg/L of filtrate. The corresponding numbers of subjects in the three Cd excretion levels were 82, 523 and 131, respectively.

In model 1, Table 3, other demographic variables were also associated with eGFR, but only in the subset with the lowest $\log(E_{Cd}/E_{cr})$. As $\log(E_{Cd}/E_{cr})$ rose, standardized β became more negative and its absolute value increased for the regression of eGFR on $\log(E_{Cd}/E_{cr})$. In contrast, standardized β remained positive for the regression of eGFR on $\log(E_{NAG}/E_{cr})$. In the subset with the highest $\log(E_{Cd}/E_{cr})$, neither of the regressions reached statistical significance. Variation in eGFR accounted for by all independent variables (adjusted R^2) *fell* as $\log(E_{Cd}/E_{cr})$ rose and eGFR fell.

In model 2, Table 3, age was the only demographic factor associated with eGFR. As $\log(E_{Cd}/C_{cr})$ rose, standardized β became more negative and its absolute value increased for regressions of eGFR on $\log(E_{Cd}/C_{cr})$ and $\log(E_{NAG}/C_{cr})$. Both regressions were highly significant in the middle and highest subsets of $\log(E_{Cd}/C_{cr})$. Variation in eGFR accounted for by all independent variables (adjusted R^2) *rose* as $\log(E_{Cd}/C_{cr})$ rose and GFR fell.

2.2. Graphic Analyses

Figure 1 compares linear and quadratic regressions of eGFR on $\log(E_{Cd}/E_{cr})$ (graph (**A**)) and $\log(E_{Cd}/C_{cr})$ (graph (**C**)). The graphs show that R^2 was higher and p was lower for regressions of eGFR on $\log(E_{Cd}/C_{cr})$. Tables (**B**) and (**D**) enumerate R^2 and unstandardized and standardized β for linear regressions in subsets defined by increasing lower limits of $\log(E_{Cd}/E_{cr})$ or $\log(E_{Cd}/C_{cr})$. In all subsets and in the sample as a whole, R^2 and absolute values of unstandardized and standardized β were higher for regressions of eGFR on $\log(E_{Cd}/C_{cr})$.

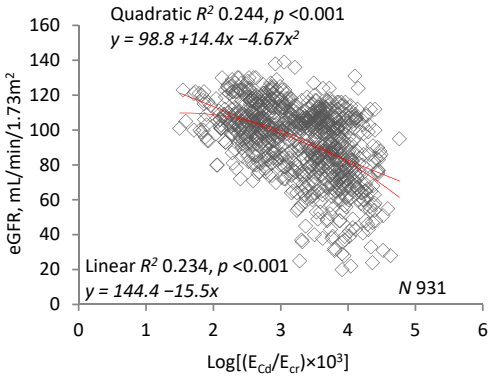
Figure 1E depicts the mean eGFR of women and men in each of three subsets defined by ranges of $\log(E_{Cd}/E_{cr})$. The ranges are those employed to denote levels of Cd excretion in Table 3, model 1. After adjustment for covariates and interactions, a significant difference in mean eGFR was demonstrated between women in the highest and lowest subsets. No differences were found among men. Figure 1F depicts the mean eGFR of women and men in each of three subsets defined by ranges of $\log(E_{Cd}/C_{cr})$. The ranges are those employed to denote levels of Cd excretion in Table 3, model 2. After adjustment for covariates and interactions, significant differences in mean eGFR were demonstrated between both women and men in the lowest subset and their counterparts in the middle and highest subsets.

Figure 2 compares linear and quadratic regressions of eGFR on $\log(E_{NAG}/E_{cr})$ (graph (**A**)) and $\log(E_{NAG}/C_{cr})$ (graph (**C**)). The graphs show that R^2 was higher and p was lower for regressions of eGFR on $\log(E_{NAG}/C_{cr})$. Tables (**B**) and (**D**) enumerate R^2 and unstandardized and standardized β for linear regressions in subsets defined by increasing lower limits of $\log(E_{NAG}/E_{cr})$ or $\log(E_{NAG}/C_{cr})$. In all subsets and in the sample as a whole, R^2 and absolute values of unstandardized and standardized β were higher for regressions of eGFR on $\log(E_{NAG}/C_{cr})$. In two subsets, only the regressions of eGFR on $\log(E_{NAG}/C_{cr})$ were significant.

Figure 2E depicts the mean eGFR of women and men in each of three subsets defined by ranges of $\log(E_{NAG}/E_{cr})$. After adjustment for covariates and interactions, a significant difference was demonstrated between men in the highest and lowest subsets. No differences in eGFR were seen among women. Figure 2F depicts the mean eGFR of women and men in each of three subsets defined by $\log(E_{NAG}/C_{cr})$. After adjustment for covariates and interactions, significant differences were demonstrated between both women and men in the lowest subset and their counterparts in the middle and highest subsets.

Figure 3 compares linear and quadratic regressions of $\log(E_{NAG}/E_{cr})$ on $\log(E_{Cd}/E_{cr})$ (graph (**A**)) or $\log(E_{NAG}/C_{cr})$ on $\log(E_{Cd}/C_{cr})$ (graph (**C**)). The graphs show that R^2 was higher and p was lower for regressions of $\log(E_{NAG}/C_{cr})$ on $\log(E_{Cd}/C_{cr})$. Tables (**B**) and (**D**) examine R^2 and unstandardized and standardized β in subsets defined by progressively increasing lower limits of $\log(E_{Cd}/E_{cr})$ or $\log(E_{Cd}/C_{cr})$. In all but one of the subsets and in the sample as a whole, R^2 and the absolute values of unstandardized and standardized β were higher for regressions of $\log(E_{NAG}/C_{cr})$ on $\log(E_{Cd}/C_{cr})$. Regressions of $\log(E_{NAG}/C_{cr})$ on $\log(E_{Cd}/C_{cr})$ were significant in three of the subsets, but regressions of $\log(E_{NAG}/E_{cr})$ on $\log(E_{Cd}/E_{cr})$ were significant in only one.

Figure 3E depicts $\log(E_{NAG}/C_{cr})$ of women and men in each of three subsets defined by ranges of $\log(E_{Cd}/E_{cr})$. After adjustment for covariates and interactions, a significant difference in mean $\log(E_{NAG}/E_{cr})$ was demonstrated between women in the highest and lowest subsets, and between men in the lowest and the middle and highest subsets. Figure 3F depicts $\log(E_{NAG}/C_{cr})$ of women and men in each of three subsets defined by ranges of $\log(E_{Cd}/C_{cr})$. After adjustment for covariates and interactions, significant differences in mean $\log(E_{NAG}/C_{cr})$ were demonstrated between both women and men in the lowest subset and their counterparts in the middle and highest subsets.

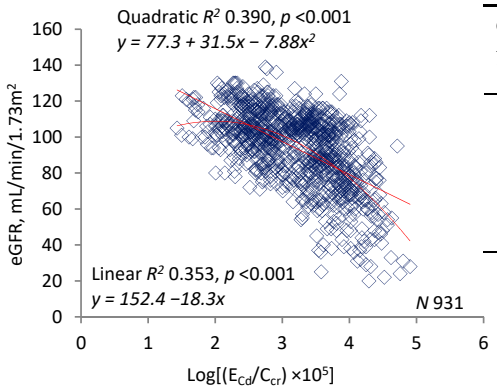


(**A**)

Cd excretion rate Log[(E$_{Cd}$/E$_{cr}$) × 10^3]	*n*	eGFR vs. log[(E$_{Cd}$/E$_{cr}$) × 10^3]			
		Unstandardized β (SE)	Standardized β	R^2	*p*
< 2.5	112	−4.86 (5.32)	−0.087	0.008	0.362
≥ 2.5	819	−17.8 (1.21)	−0.456	0.208	<0.001
≥ 3.0	603	−19.4 (2.16)	−0.343	0.118	<0.001
≥ 3.5	427	−24.2 (3.72)	−0.301	0.091	<0.001
≥ 4.0	145	−26.5 (10.7)	−0.202	0.041	0.015
1.5–4.8	931	−15.5 (0.92)	−0.484	0.234	<0.001

The unstandardized β coefficient indicates the slope. The standardized β coefficient indicates the strength of the association of eGFR with log[(E$_{Cd}$/E$_{cr}$) × 10^3]. Log [(E$_{Cd}$/E$_{cr}$) × 10^3] of 2.5, 3.0, 3.5 and 4.0 correspond to E$_{Cd}$/E$_{cr}$ of 0.28, 0.89, 2.87 and 8.93 μg/g creatinine, respectively.

(**B**)

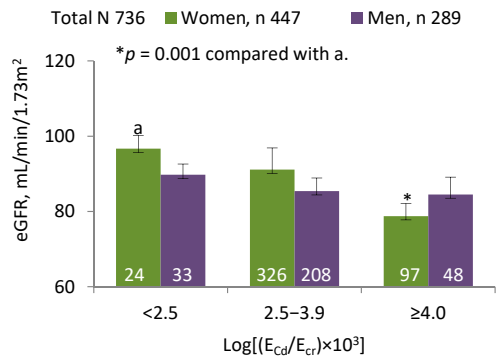


(**C**)

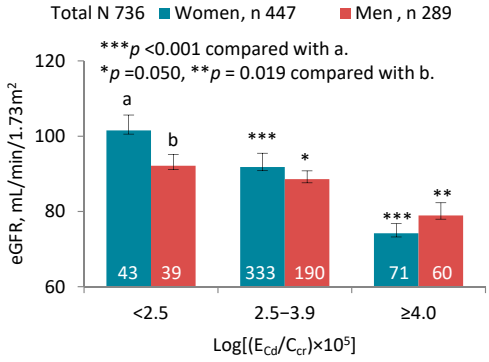
Cd excretion rate Log[(E$_{Cd}$/c$_{cr}$) × 10^5]	*n*	eGFR vs. log[(E$_{Cd}$/c$_{cr}$) × 10^5]			
		Unstandardized β (SE)	Standardized β	R^2	*p*
< 2.5	146	−1.74 (4.13)	−0.035	0.001	0.674
≥ 2.5	785	−21.5 (1.13)	−0.502	0.316	<0.001
≥ 3.0	567	−27.7 (1.94)	−0.514	0.264	<0.001
≥ 3.5	373	−34.1 (3.30)	−0.473	0.223	<0.001
≥ 4.0	131	−44.0 (8.63)	−0.409	0.168	<0.001
1.5–4.9	931	−18.3 (0.32)	−0.594	0.353	<0.001

The unstandardized β coefficient indicates the slope. The standardized β coefficient indicates the strength of the association of eGFR with log[(E$_{Cd}$/C$_{cr}$)×10^5]. Log [(E$_{Cd}$/C$_{cr}$)×10^5] of 2.5, 3.0, 3.5 and 4.0 correspond to E$_{Cd}$/C$_{cr}$×100 of 0.28, 0.91, 2.83 and 8.92 μg/L, respectively.

(**D**)

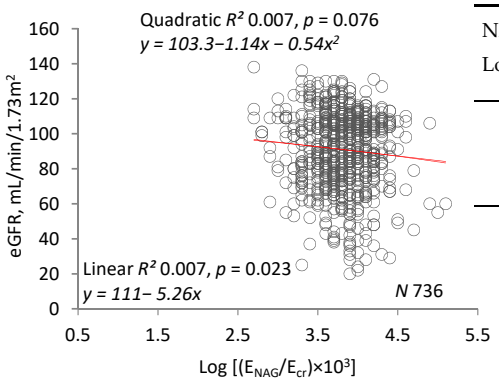


(**E**)



(**F**)

Figure 1. Inverse relationships of eGFR to parameters of Cd excretion. Parameter abbreviations are summarized at the end of the text. Scatterplots in graphs (**A**,**C**) relate eGFR to log[(E$_{Cd}$/E$_{cr}$) × 10^3] and log[(E$_{Cd}$/C$_{cr}$) × 10^5] in all subjects. Linear and quadratic equations, their respective coefficients of determination (R^2), and associated *p*-values are provided. In Table (**B**), linear relationships of eGFR to log[(E$_{Cd}$/E$_{cr}$) × 10^3] < 2.5, ≥2.5, ≥3.0, ≥3.5 and ≥4.0 are characterized with respective values of subject number (*n*), unstandardized and standardized β, R^2, and *p*. In Table (**D**, linear relationships of eGFR to log[(E$_{Cd}$/C$_{cr}$) × 10^5] of <2.5, ≥2.5, ≥3.0, ≥3.5 and ≥4.0 are characterized with respective values of subject number (*n*), unstandardized and standardized β, R^2, and *p*. In graphs (**E**,**F**), bars represent mean eGFR in women and men grouped by ranges of log[(E$_{Cd}$/E$_{cr}$) × 10^3] or log[(E$_{Cd}$/C$_{cr}$) × 10^5]. The subsets thus created are identical to those constituting Cd excretion levels 1, 2, and 3 in Table 3. The letters a and b identify reference values in women and men, respectively, at the lowest rates of Cd excretion. Where appropriate, statistical comparisons are made within each gender between mean eGFR in bars a and b and mean eGFR at higher rates of Cd excretion. Geometric mean (GM) values (standard deviation, SD) of E$_{Cd}$/E$_{cr}$ are 0.15 (0.07) μg/g creatinine at level 1, 2.35 (2.42) μg/g creatinine at level 2, and 14.91(7.57) μg/g creatinine at level 3. GM (SD) of [(E$_{Cd}$/C$_{cr}$) × 100] are 0.15 (0.08) μg/L at level 1, 2.20 (2.25) μg/L at level 2, and 16.01 (11.05) μg/L at level 3.

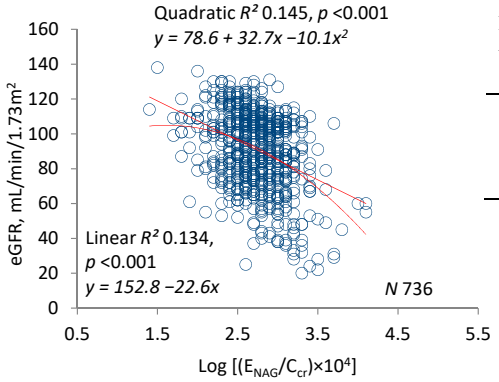


(A)

NAG excretion rate Log[(E$_{NAG}$/E$_{cr}$) × 10^3]	*n*	eGFR vs. log[(E$_{NAG}$/E$_{cr}$) × 10^3]			
		Unstandardized β (SE)	Standardized β	R^2	*p*
< 3.5	101	−14.0 (11.8)	−0.118	0.014	0.238
≥ 3.5	635	−5.36 (2.38)	−0.065	0.004	0.103
≥ 4.0	244	−9.77 (7.38)	−0.085	0.003	0.187
2.7–5.1	736	−5.37 (2.37)	−0.084	0.006	0.023

The unstandardized β coefficient indicates the slope. The standardized β coefficient indicates the strength of the association of eGFR with log[(E$_{NAG}$/E$_{cr}$) × 10^3]. Log [(E$_{NAG}$/E$_{cr}$) × 10^3] of 3.5 and 4.0 correspond to E$_{NAG}$/E$_{cr}$ of 2.83 and 8.98 units/g creatinine, respectively.

(B)

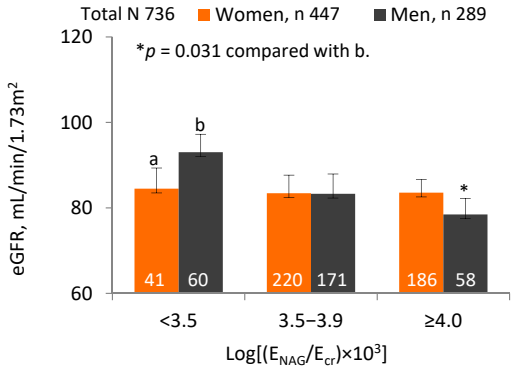


(C)

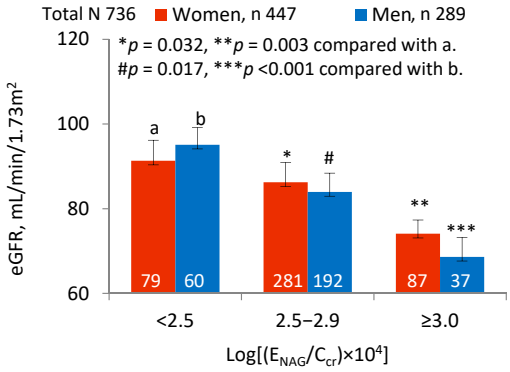
NAG excretion rate Log[(E$_{NAG}$/C$_{cr}$) × 10^4]	*n*	eGFR vs. log[(E$_{NAG}$/C$_{cr}$) × 10^4]			
		Unstandardized β (SE)	Standardized β	R^2	*p*
< 2.5	139	−10.5 (7.97)	−0.112	0.013	0.189
≥ 2.5	597	−27.9 (3.16)	−0.341	0.116	<0.001
≥ 3.0	191	−36.2 (8.36)	−0.301	0.090	<0.001
1.4–4.1	736	−22.5 (2.14)	−0.362	0.131	<0.001

The unstandardized β indicates the slope. The standardized β coefficient indicates the strength of the association of eGFR with log[(E$_{NAG}$/C$_{cr}$) × 10^4]. Log [(E$_{NAG}$/C$_{cr}$) × 10^4] of 2.5 and 3.0 correspond to E$_{NAG}$/C$_{cr}$ ×100 of 2.82 and 8.97 units/L, respectively.

(D)

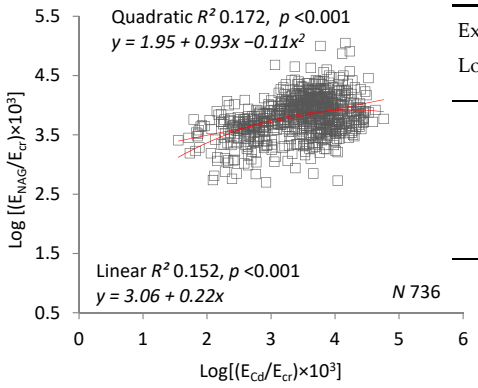


(E)



(F)

Figure 2. Inverse relationships of eGFR to parameters of NAG excretion. Parameter abbreviations are summarized at the end of the text. Scatterplots in graphs (**A**,**C**) relate eGFR to log[(E$_{NAG}$/E$_{cr}$) × 10^3] and log[(E$_{NAG}$/C$_{cr}$) × 10^4] in all subjects. Linear and quadratic equations, their respective coefficients of determination (R^2), and associated *p*-values are provided. In Table (**B**), linear relationships of eGFR to log [(E$_{NAG}$)/E$_{cr}$) × 10^3] < 3.5, ≥3.5 and ≥4.0 are characterized with respective values of subject number (*n*), unstandardized and standardized β, R^2, and *p*. In Table (**D**), linear relationships of eGFR to log[(E$_{NAG}$/C$_{cr}$) × 10^4] < 2.5, ≥2.5 and ≥3.0 are characterized with respective values of *n*, unstandardized and standardized β, R^2, and *p*. In graphs (**E**,**F**), the bars represent the mean eGFR in women and men grouped by ranges of log[(E$_{NAG}$/E$_{cr}$) × 10^3] or log[(E$_{NAG}$/C$_{cr}$) × 10^4] (NAG excretion levels 1, 2, and 3 from lowest to highest). Numbers of women and men within each NAG excretion level are provided. The letters a and b identify reference values in women and men, respectively, at the lowest rates of Cd excretion. Where appropriate, statistical comparisons are made within each gender between mean eGFR in bars a and b and mean eGFR at higher rates of Cd excretion. The geometric mean (GM) (SD) of E$_{NAG}$/E$_{cr}$ in groups 1, 2 and 3 is 1.75 (0.65), 5.36 (1.68) and 14.50 (11.98) units/g creatinine, respectively. The GM (SD) of [(E$_{NAG}$/C$_{cr}$) × 100] in groups 1, 2 and 3 is 1.73 (0.66), 5.56 (2.25) and 17.52 (17.16) units/L, respectively.

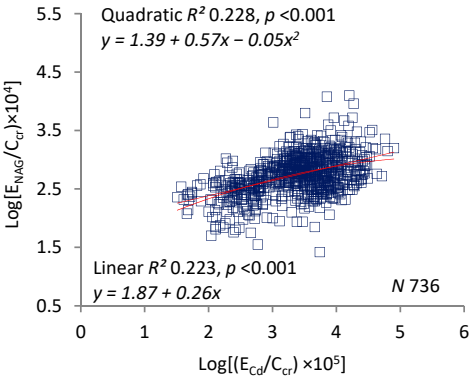


(A)

Excretion rate Log[(E_{Cd}/E_{cr}) × 10³]	*n*	Log[(E_{NAG}/E_{cr}) × 10³ vs. log[(E_{Cd}/E_{cr}) × 10³]			
		Unstandardized β (SE)	Standardized β	R^2	*p*
< 2.5	57	0.05 (0.19)	0.038	0.001	0.778
≥ 2.5	679	0.19 (0.02)	0.296	0.208	<0.001
≥ 3.0	565	0.04 (0.04)	0.051	0.003	0.227
≥ 3.5	425	0.01 (0.06)	0.007	0.000	0.891
≥ 4.0	145	0.21 (0.16)	0.107	0.011	0.199
1.6–4.8	736	0.22 (0.02)	0.390	0.152	<0.001

The unstandardized β coefficient indicates the slope. The standardized β coefficient indicates the strength of the association of log[(E_{NAG}/E_{cr}) × 10³] with log[(E_{Cd}/E_{cr}) × 10³]. Log [(E_{Cd}/E_{cr}) × 10³] of 2.5, 3.0, 3.5 and 4.0 correspond to E_{Cd}/E_{cr} of 0.28, 0.89, 2.87 and 8.93 μg/g creatinine, respectively.

(B)

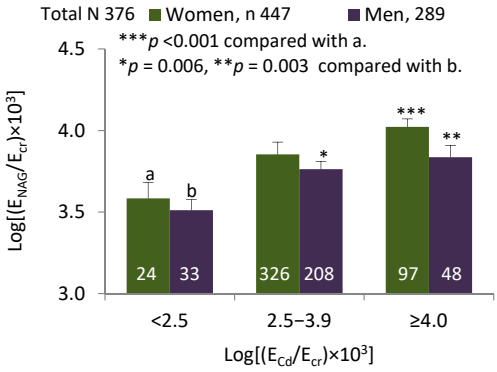


(C)

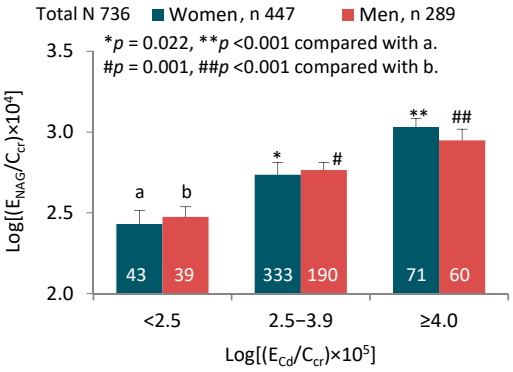
Excretion rate Log[(E_{Cd}/C_{cr})× 10⁵]	*n*	Log[(E_{NAG}/C_{cr}) × 10⁴ vs. log[(E_{Cd}/C_{cr}) × 10⁵]			
		Unstandardized β (SE)	Standardized β	R^2	*p*
< 2.5	82	0.14 (0.12)	0.122	0.015	0.275
≥ 2.5	654	0.24 (0.02)	0.361	0.130	<0.001
≥ 3.0	548	0.13 (0.04)	0.159	0.025	<0.001
≥ 3.5	374	0.21 (0.06)	0.189	0.036	<0.001
≥ 4.0	131	0.26 (0.14)	0.157	0.025	0.073
1.5–4.9	736	0.26 (0.02)	0.474	0.223	<0.001

The unstandardized β coefficient indicates the slope. The standardized β coefficient indicates the strength of the association of log[(E_{NAG}/C_{cr}) × 10⁴] with log[(E_{Cd}/C_{cr}) × 10⁵]. Log [(E_{Cd}/C_{cr}) × 10⁵] of 2.5, 3.0, 3.5 and 4.0 correspond to E_{Cd}/C_{cr} × 100 of 0.28, 0.91, 2.83 and 8.92 μg/L, respectively.

(D)



(E)



(F)

Figure 3. Direct relationships of parameters of NAG excretion to parameters of Cd excretion. Parameter abbreviations are summarized at the end of the text. Scatterplots in graphs (**A**,**C**) relate log[(E_{NAG}/E_{cr}) × 10^3] to log[(E_{Cd}/E_{cr}) × 10^3], and log[(E_{NAG}/C_{cr}) × 10^4] to log[(E_{Cd}/C_{cr}) × 10^5] in all subjects. Linear and quadratic equations, their respective coefficients of determination (R^2), and associated *p*-values are provided. In Table (**B**), linear relationships of log[(E_{NAG}/E_{cr}) × 10^3] to log [(E_{Cd}/E_{cr}) × 10^3] < 2.5, ≥2.5, ≥3.0, ≥3.5 and ≥4.0 are characterized with respective values of subject number (*n*), unstandardized and standardized β, R^2, and *p*. In Table (**D**), linear relationships of log[(E_{NAG}/C_{cr}) × 10^4] to log [(E_{Cd}/C_{cr}) × 10^5] < 2.5, ≥2.5, ≥3.0, ≥3.5 and ≥4.0 are characterized with respective values of subject number (*n*), unstandardized and standardized β, R^2, and *p*. In graphs (**E**,**F**), the bars represent mean log[(E_{NAG}/E_{cr}) × 10^3] or mean log[(E_{NAG}/C_{cr}) × 10^5] in women and men grouped by ranges of log[(E_{Cd}/E_{cr}) × 10^3] or log[(E_{Cd}/C_{cr}) × 10^5]. The subsets thus created are identical to those constituting Cd excretion levels in Table 3 and in Figure 1E,F. Numbers of subjects within each Cd excretion level and corresponding values of GM (SD) of E_{Cd}/E_{cr} and [(E_{Cd}/C_{cr}) × 100] are as described in the legend for Figure 1. The letters a and b identify reference values in women and men, respectively, at the lowest rates of Cd excretion. Where appropriate, statistical comparisons are made within each gender between mean eGFR in bars a and b and mean eGFR at higher rates of Cd excretion.

3. Discussions

We have previously argued that regressions of eGFR on parameters of Cd and NAG excretion provide insight into the pathogenesis of Cd nephropathy [15]. Heretofore, investigators of this issue have normalized $[Cd]_u$ and $[NAG]_u$ to $[cr]_u$ [7,8,16,17]. The resulting ratios adjust $[Cd]_u$ and $[NAG]_u$ for variation in V_u, but muscle mass affects $[cr]_u$, and nephron number affects excretion rates of substances that emanate from tubular cells. In theory, normalization of E_{Cd} and E_{NAG} to C_{cr} resolves these issues for reasons summarized in the Introduction. We therefore hypothesized that this methodological modification would add clarity to relationships among eGFR, E_{Cd}, and E_{NAG}.

3.1. Interpretation of Tabular Analyses

We created the study sample to encompass a broad range of probability that asymptomatic participants had developed Cd nephropathy. To highlight the relevance of previous Cd accumulation to GFR, we divided the sample into three subsets defined by gradations of $\log(E_{Cd}/C_{cr})$. As $\log(E_{Cd}/C_{cr})$ rose, age, E_{Cd}/E_{cr}, E_{NAG}/E_{cr}, E_{Cd}/C_{cr}, and E_{NAG}/C_{cr} also rose, and eGFR fell (Table 1).

We performed two multivariable logistic regression analyses to quantify effects of demographic features and excretory parameters on the probability of CKD (eGFR < 60 $mL/min/1.73m^2$) (Table 2). The unit of probability was the ratio of odds of having CKD to odds of not having it, i.e., the probability odds ratio (POR). POR was associated with $\log_2(E_{Cd}/E_{cr})$ and $\log_2(E_{NAG}/E_{cr})$ in model 1 and with $\log_2(E_{Cd}/C_{cr})$ and $\log_2(E_{NAG}/C_{cr})$ in model 2. POR was higher for logs of the ratios in model 2, as were the percentage increases in POR per doubling of ratios.

We also performed two regression model analyses of eGFR on demographic variables and parameters of Cd and NAG excretion (Table 3). Each regression was examined in three subsets defined by progressively higher ranges of $\log(E_{Cd}/E_{cr})$ (model 1) or $\log(E_{Cd}/C_{cr})$ (model 2). The ranges were designated Cd excretion levels 1, 2, and 3. In model 1, associations of eGFR with $\log(E_{Cd}/E_{cr})$ and $\log(E_{NAG}/E_{cr})$ became *less* significant as $\log(E_{Cd}/E_{cr})$ rose in the subsets. Moreover, at excretion level 3, standardized β for the regression of eGFR on E_{NAG}/E_{cr} was positive rather than negative, and thus implied the absence of an inverse relationship between eGFR and the severity of tubular injury. In model 2, as $\log(E_{Cd}/C_{cr})$ rose in the Cd excretion subsets, associations of eGFR with E_{Cd}/C_{cr} and E_{NAG}/C_{cr} became *more* significant, the absolute value of standardized β (strength of association) increased, the slope implied by standardized β became more negative, the inverse relationship of eGFR to tubular injury was thus enhanced, and R^2 for the entire analysis rose.

Collectively, our tabular data demonstrate multiple benefits of normalizing excretion rates to C_{cr} rather than E_{cr}. This approach substantially magnified the effects of E_{Cd} and E_{NAG} on POR for CKD; exposed the inverse relationship between eGFR and E_{NAG} at high levels of Cd excretion; demonstrated qualitatively similar regressions of eGFR on $\log(E_{Cd}/C_{cr})$ and $\log(E_{NAG}/C_{cr})$; increased the size of effects of E_{Cd} and E_{NAG} on eGFR and the strength of associations among these variables; and produced a multilinear regression model that accounted for more variation in eGFR.

3.2. Interpretation of Graphic Analyses

The univariate analyses in Figures 1 and 2 confirm the concepts imparted by Table 3. Figure 1 depicts linear and quadratic regressions of eGFR on $\log(E_{Cd}/E_{cr})$ or $\log(E_{Cd}/C_{cr})$. R^2 was higher and *p* was lower for both types of regression when eGFR was plotted against $\log(E_{Cd}/C_{cr})$; moreover, dispersion was visibly reduced and a curvilinear relationship between eGFR and E_{Cd} was more evident (Figure 1A,C). Slope analyses were performed over ranges of $\log(E_{Cd}/E_{cr})$ or $\log(E_{Cd}/C_{cr})$ that were progressively reduced by raising lower limits. Within each range, effect size (unstandardized β) and strength of association (standardized β) were greater for relationships of eGFR to $\log(E_{Cd}/C_{cr})$. Subsets created in Table 3 according to Cd excretion level were employed in Figure 1E,F, and differences in mean eGFR were more pronounced among subsets defined by $\log(E_{Cd}/C_{cr})$.

Figure 2 depicts linear and quadratic regressions of eGFR on $\log(E_{NAG}/E_{cr})$ or $\log(E_{NAG}/C_{cr})$. R^2 was much higher for both regressions when eGFR was plotted against $\log(E_{NAG}/C_{cr})$; simultaneously, dispersion was reduced, and both linear and quadratic relationships of eGFR to E_{NAG} were more visually evident. As in Figure 1, slope analyses were performed over ranges of $\log(E_{Cd}/E_{cr})$ or $\log(E_{Cd}/C_{cr})$ that were progressively reduced by raising lower limits. Within each range, effect size (unstandardized β) and strength of association (standardized β) were much greater for relationships of eGFR to $\log(E_{NAG}/C_{cr})$. Linear regressions relevant to each range were significant only for the highest range of E_{NAG}/E_{cr}, but they were highly significant for all but the lowest range of E_{NAG}/C_{cr}. Three subsets were created within the sample according to low, medium, or high ranges of $\log(E_{NAG}/E_{cr})$ or $\log(E_{NAG}/C_{cr})$; as in Figure 1, differences in mean eGFR were more pronounced among subsets defined by $\log(E_{NAG}/C_{cr})$.

Figure 3 depicts linear and quadratic regressions of $\log(E_{NAG}/E_{cr})$ on $\log(E_{Cd}/E_{cr})$, and $\log(E_{NAG}/C_{cr})$ on $\log(E_{Cd}/C_{cr})$. Although the graphs are visually similar, a reduction in dispersion was demonstrated in the plot of E_{NAG}/C_{cr} against E_{Cd}/C_{cr}, and R^2 was higher for both linear and quadratic regressions of $\log(E_{NAG}/C_{cr})$ on $\log(E_{Cd}/C_{cr})$. In the slope analyses, R^2 and unstandardized and standardized β and were higher for each individual linear regression of $\log(E_{NAG}/C_{cr})$ on $\log(E_{Cd}/C_{cr})$. As in Figure 1, subsets created in Table 3 according to Cd excretion level were employed in Figure 3E,F. Differences in NAG excretion were more pronounced when subsets were defined by $\log(E_{Cd}/C_{cr})$. Thus, in all three figures, normalization of excretion rates to C_{cr} rather than E_{cr} increased coefficients of determination, effect size, and strength of association for each possible bivariate relationship among eGFR, E_{Cd}, and E_{NAG}. Within each gender, normalization to C_{cr} also accentuated differences in eGFR and NAG excretion among subsets defined by Cd excretion.

3.3. Creatinine Excretion, Creatinine Clearance, and GFR

Creatinine, a small nitrogenous waste product (m.w. 113 Da), is synthesized from creatine phosphate in skeletal muscle. It can enter plasma from the gut if meat is ingested, but most of its flux into plasma results from endogenous production in muscle cells. A small fraction of the influx is diverted to the colonic lumen for bacterial metabolism, and that fraction increases as $[cr]_p$ rises. Nevertheless, at all but the most severely reduced values of GFR, renal excretion is the principal avenue of creatinine elimination [25,26].

When plasma is in equilibrium with respect to creatinine, the principal determinant of E_{cr} is muscle mass, which is highly variable in the population [18]. Most excreted creatinine is filtered, and the remainder is secreted by proximal tubules. The secreted fraction is small when GFR is normal; however, as GFR falls, the secreted fraction rises, C_{cr} ($=E_{cr}/[cr]_p$) increasingly overestimates GFR [26,27], and in theory, E_{Cd}/C_{cr} and E_{NAG}/C_{cr} increasingly underestimate the excretion of Cd and NAG per volume of filtrate. If C_{cr} were more uniformly representative of GFR, the relationships of eGFR to $\log(E_{Cd}/C_{cr})$ and $\log(E_{NAG}/C_{cr})$ would likely be less quadratic and more linear than Figures 1 and 2 indicate. In any case, the inverse nature of these relationships is indisputable.

Estimated GFR approximates the radionuclide-based gold standard of GFR determination more closely than C_{cr} does [24]. One could argue, therefore, that we should normalize E_{Cd} and E_{NAG} to eGFR in our work. In theory, this method would offer the same advantages as normalization to C_{cr}, and at low GFR, it would prevent underestimation of E_{Cd} and E_{NAG} per volume of filtrate. The practical obstacle to this approach is that determinations of E_{Cd}/eGFR and E_{NAG}/eGFR would require timed urine collections for the measurement of E_{Cd} and E_{NAG}. Normalization to C_{cr}, though possibly less accurate, is more convenient and less susceptible to procedural error because it is accomplished with single aliquots of serum and urine.

3.4. Tubular Release of Cd and NAG Necessitates Normalization of Excretion Rates to C_{cr}

Excreted NAG emanates exclusively from injured tubular cells because the molecule is too large to undergo glomerular filtration [4]. The source of excreted Cd is more debatable, but several considerations suggest that Cd is also released from injured cells [15]. In multiple studies, E_{Cd} varied directly, not inversely, with GFR (nephron number) [19–21]. Similarly, E_{Cd} varied directly with the Cd content of kidneys sampled at autopsy or transplantation [28,29]. Because animal studies indicated that the tubular reabsorptive capacity for filtered Cd is quite high [30], it is unlikely that a typically intoxicated human excretes unreabsorbed Cd immediately after filtration. Previously reported correlations of E_{Cd} with E_{NAG} suggest that Cd and NAG emanate from a common source [5–15]; consequently, we have argued that both Cd and NAG are released into glomerular filtrate from tubular cells. If this inference is correct, then E_{Cd}, like E_{NAG}, is an indicator of Cd-induced tubular injury [15]. Tubulointerstitial nephritis, destruction of nephrons, and a reduction in GFR are logical sequelae of such injury [3,31].

If urinary Cd and NAG emanate from tubules, then we should expect the excretion of these substances to vary directly with the number of intact nephrons and the severity of cellular injury. In the absence of renal hypoperfusion, we assume a proportional relationship between GFR and nephron number [3]. Consequently, to focus on the severity of injury as a determinant of eGFR, we nullify the simultaneous contribution of nephron number to E_{Cd} and E_{NAG} by normalizing these excretion rates to C_{cr}. Whereas the relationship of E_{cr} to nephron number is indirect and highly variable among subjects, the relationship of C_{cr} to nephron number is relatively direct and consistent. On theoretical grounds, we expect eGFR to be more closely associated with E_{Cd}/C_{cr} and E_{NAG}/C_{cr} than with E_{Cd}/E_{cr} and E_{NAG}/E_{cr}, and the data reported herein confirm that expectation. We recommend the adoption of C_{cr} as the optimal denominator for the normalization of excretion rates in studies of Cd nephropathy.

4. Materials and Methods

4.1. Study Population

To develop a diverse sample with a wide range of environmental exposure to Cd, we assembled archived data drawn from multiple sites in Thailand. At the time of recruitment, all participants had lived at their current addresses for at least 30 years, and all gave informed consent to participate. Exclusion criteria were pregnancy, breast-feeding, a history of metal work, and a hospital record or physician's diagnosis of an advanced chronic disease. Smoking, diabetes, hypertension, regular use of medications, educational level, occupation, and family health history were ascertained by questionnaire. Diabetes was defined as fasting plasma glucose levels $\geq$126 mg/dL or a physician's prescription of anti-diabetic medications. Hypertension was defined as systolic blood pressure $\geq$140 mmHg, diastolic blood pressure $\geq$90 mmHg, a physician's diagnosis, or prescription of anti-hypertensive medications.

Blood and urine were obtained in 2001 and 2002 from control subjects in Bangkok, and in 2004 and 2005 from subjects in subsistence farming areas of Mae Sot District. As judged by the Cd content of rice, a dietary staple in Thailand, exposure to Cd was low in Bangkok and moderate or high in Mae Sot [17,32–34]. Because occupational exposure was an exclusion criterion, we presumed that all participants had acquired Cd from the environment. After exclusion of participants with incomplete datasets, the study sample included 545 women and 386 men. Within the sample, urinary Cd varied by a factor >1000; age ranged from 16 to 87 years, and eGFR from 20 to 139 mL/min/1.73 m^2. The Institutional Ethical Committees of Chulalongkorn University, Chiang Mai University, and the Mae Sot Hospital approved the study protocol [33].

4.2. Specimen Collection and Analysis

Second morning-void urine samples were collected after an overnight fast. Within the ensuing 3 h, 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 or −80 °C for later analysis. Assays of creatinine in urine and serum ($[cr]_u$, $[cr]_p$) were based on the Jaffe reaction. The assay of NAG in urine ($[NAG]_u$) was based on colorimetry (NAG test kit, Shionogi Pharmaceuticals, Sapporo, Japan).

For the Bangkok group, $[Cd]_u$ was determined by inductively-coupled plasma mass spectrometry (ICP/MS, Agilent 7500, Agilent Technologies, Santa Clara, CA, USA) because this method was sufficiently sensitive 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, and accuracy and precision of those analyses were evaluated with reference urine (Lyphochek®, Bio-Rad, Gladesville, New South Wales, Australia). When $[Cd]_u$ was less than the detection limit, 0.05 μg/L, the concentration assigned was the detection limit divided by the square root of 2. Fifty-eight subjects (14.7%) in the Bangkok group had $[Cd]_u < 0.05$ μg/L.

For the Mae Sot groups, $[Cd]_u$ 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 $[Cd]_u$ below the detection limit.

4.3. Normalization of Excretion Rates to E_{cr} and C_{cr}

Excretion rates of Cd and NAG (E_{Cd} and E_{NAG}) were normalized to E_{cr} or to C_{cr}, a surrogate for GFR. E_{Cd}/E_{cr} and E_{NAG}/E_{cr} were expressed as mass excreted per g of creatinine; E_{Cd}/C_{cr} and E_{NAG}/C_{cr} were expressed as mass excreted per volume of filtrate. For x = Cd or NAG, E_x/E_{cr} was calculated as $[x]_u/[cr]_u$, and E_x/C_{cr} was calculated as $[x]_u[cr]_p/[cr]_u$ [23].

4.4. Estimated Glomerular Filtration Rates (eGFR)

The glomerular filtration rate was estimated with equations from the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) [27]. CKD stages 1, 2, 3, 4, and 5 corresponded to eGFR of 90–119, 60–89, 30–59, 15–29 and <15 mL/min/1.73 m^2, respectively. For dichotomous comparisons, CKD was defined as eGFR < 60 mL/min/1.73 m^2.

4.5. Statistical Analysis

Data were analyzed with SPSS 17.0 (SPSS Inc., Chicago, IL, USA). The Kruskal–Wallis test was used to assess differences in means among three subsets, and the Pearson chi-squared test was used to assess differences in percentages. The one-sample Kolmogorov–Smirnov test was used to identify departures of continuous variables from a normal distribution, and a base-10 or base-2 logarithmic transformation was applied to variables that showed rightward skewing before they were subjected to parametric statistical analysis. Throughout the text, base-10 and base-2 logarithms are denoted as $\log(x)$ and $\log_2(x)$, respectively.

In our regression analyses (Tables 2 and 3), independent variables included $\log(E_{Cd}/E_{cr})$, $\log(E_{Cd}/C_{cr})$, $\log(E_{NAG}/E_{cr})$, $\log(E_{NAG}/C_{cr})$, age, diabetes, gender, hypertension, and smoking. For each independent variable (x), we used multivariable logistic regression analyses to ascertain the prevalence odds ratio (POR) for CKD and the corresponding β-coefficient (Table 2). The β-coefficient—i.e., the slope of a line relating the natural log of POR to x—thus depicted the effect of a one-unit change in x on POR while other independent variables remained constant. Log base-2 transformation of E_{Cd}/E_{cr}, E_{NAG}/E_{cr}, E_{Cd}/C_{cr}, and E_{NAG}/C_{cr} permitted estimation of the factor by which POR increased as each ratio was doubled. We employed two models in each logistic regression analysis: model 1 incorporated $\log_2(E_{Cd}/E_{cr})$ and $\log_2(E_{NAG}/E_{cr})$; model 2 incorporated $\log_2(E_{Cd}/C_{cr})$ and $\log_2(E_{NAG}/C_{cr})$.

We performed multivariable linear regression analyses in three subsets of the study sample (Table 3). The subsets were defined by gradations of $\log[(E_{Cd}/E_{cr}) \times 10^3]$ (model 1)

or $\log[(E_{Cd}/C_{cr}) \times 10^5]$ (model 2). We examined associations of eGFR with $\log_{10}(E_{Cd}/E_{cr})$, $\log_{10}(E_{NAG}/E_{cr})$, $\log_{10}(E_{Cd}/C_{cr})$, $\log_{10}(E_{NAG}/C_{cr})$, and the aforementioned demographic variables. For each model, an adjusted coefficient of determination (R^2) and standardized β were obtained to indicate, respectively, the total variation in eGFR that was explained by all independent variables, and the strength of association between eGFR and an individual independent variable.

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

Relationships between x and y were assessed with R^2 (the coefficient of determination) and with unstandardized and standardized β coefficients. In linear and quadratic models, R^2 is the fraction of variation in y that is explained by variation in x. In linear models, the unstandardized β coefficient is the slope of the linear regression, and the standardized β coefficient indicates the strength of the association between y and x on a uniform scale. A linear regression method was used to perform slope analyses of quadratic curves relating eGFR to $\log(E_{Cd}/E_{cr})$, $\log(E_{NAG}/E_{cr})$, $\log(E_{Cd}/C_{cr})$, and $\log(E_{NAG}/C_{cr})$.

In Figure 1E,F, Figure 2E,F and Figure 3E,F, a univariate model analysis was used to derive mean eGFR (Figures 1 and 2), mean $\log(E_{NAG}/E_{cr})$ (Figure 3), and mean $\log(E_{NAG}/C_{cr})$ (Figure 3) for men and women separately with adjustment for covariates (including age) and interactions among independent variables. The Cd-excretion levels in subsets of Figure 1E,F and Figure 3E,F are identical to those depicted in subsets of Table 3. Raw data for eGFR were employed in Tables 1 and 2 and in Figure 1A,C and Figure 2A,C. In all analyses, two-sided p-Values ≤ 0.05 were assumed to indicate statistical significance.

5. Conclusions

Excretion rates of Cd and NAG elucidate reductions in GFR that result from renal accumulation of Cd. The conventional method for expressing these excretion rates, normalization of urine concentrations to $[cr]_u$, incorporates conceptual flaws that are eliminated if the rates are normalized to C_{cr}. In a large and diverse sample of Thai subjects, the alternate approach strengthened all identifiable relationships between eGFR and conventionally quantified urine components, and exposed additional relationships that were obscured by the conventional method. Normalization to C_{cr} should replace normalization to $[cr]_u$ in studies that relate urine composition to Cd-induced diminution of eGFR.

Author Contributions: S.S. and K.R.P. conceptualized the comparison of two methods for the normalization of excretion rates. K.R.P. proposed the normalization of excretion rates to creatinine clearance (C_{cr}). In collaboration with G.C.G. and D.A.V., S.S. organized and analyzed the data and created the tables and figures. K.R.P. wrote initial and subsequent drafts of the manuscript, and S.S. reviewed and edited all drafts. W.R. and M.N. supervised the recruitment of participants and the collection of biologic specimens in Thailand. All authors have read and agreed to the published version of the manuscript.

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Informed Consent Statement: All participants took part in the study after giving informed consent.

Data Availability Statement: Data are contained within the article.

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Abbreviations

GFR	Glomerular filtration rate, mL/min
eGFR	Estimated glomerular filtration rate, mL/min/1.73m^2 (of body surface area)
CKD-EPI	Chronic kidney disease epidemiology collaboration
Cd	Cadmium
NAG	N-acetyl-β-D-glucosaminidase
Cr	Creatinine
C_{cr}	Creatinine clearance (a surrogate for GFR), mL/min
V_u	Rate of urine flow, volume/time
$[x]_u$	Urine concentration of substance x (cr, Cd, or NAG), mass/volume
E_x	Excretion rate of substance x (cr, Cd, or NAG), mass/time
E_x/E_{cr}	Amount of x excreted per gram of creatinine excreted, mass/mass
$[x]_u/[cr]_u$	Ratio of urine concentration of x to urine concentration of cr, mass/mass
E_x/C_{cr}	Amount of x excreted per volume of filtrate, mass/volume
MT	Metallothionein
CdMT	Cd-metallothionein complex
POR	Probability odds ratio

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