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The Validity of Benchmark Dose Limit Analysis for Estimating Permissible Accumulation of Cadmium

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Abstract: Cadmium (Cd) is a toxic metal pollutant that accumulates, especially in the proximal tubular epithelial cells of kidneys, where it causes tubular cell injury, cell death and a reduction in glomerular filtration rate (GFR). Diet is the main Cd exposure source in non-occupationally exposed and non-smoking populations. The present study aimed to evaluate the reliability of a tolerable Cd intake of 0.83 μg/kg body weight/day, and its corresponding toxicity threshold level of 5.24 μg/g creatinine. The PROAST software was used to calculate the lower 95% confidence bound of the benchmark dose (BMDL) values of Cd excretion (E_{Cd}) associated with injury to kidney tubular cells, a defective tubular reabsorption of filtered proteins, and a reduction in the estimated GFR (eGFR). Data were from 289 males and 445 females, mean age of 48.1 years of which 42.8% were smokers, while 31.7% had hypertension, and 9% had chronic kidney disease (CKD). The BMDL value of E_{Cd} associated with kidney tubular cell injury was 0.67 ng/L of filtrate in both men and women. Therefore, an environmental Cd exposure producing E_{Cd} of 0.67 ng/L filtrate could be considered as Cd accumulation levels below which renal effects are likely to be negligible. A reduction in eGFR and CKD may follow when E_{Cd} rises from 0.67 to 1 ng/L of filtrate. These adverse health effects occur at the body burdens lower than those associated with E_{Cd} of 5.24 μg/g creatinine, thereby arguing that current health-guiding values do not provide a sufficient health protection.

Keywords: benchmark dose; BMD lower confidence limit; BMDL; BMD upper confidence limit; BMDU; cadmium; β_2-microglobulin; eGFR; N-acetyl-β-D-glucosaminidase; NOAEL

1. Introduction

Cadmium (Cd) is an environmental toxicant of worldwide public health significance because numerous population-based studies suggest that exposure to Cd even at low levels adversely impacts the functions of most organs of the body [1]. Volcanic emissions, biomass and fossil fuel combustion, and cigarette smoke are sources of environmental Cd pollution [2–6]. Cd in cigarette smoke as a volatile metallic form and oxide (CdO) has a particularly high transmission rate [7]. The utility of Cd in many industrial processes, and the use of phosphate fertilizers contaminated with Cd cause also a widespread dispersion of this toxic metal in the environment and subsequently the food chains [8–12].

Foods that are frequently consumed in large quantities such as rice, potatoes, wheat, leafy salad vegetables, and other cereal crops form the most significant dietary sources of Cd [13–15]. Seafood (shellfish), molluscs and crustaceans are additional dietary Cd sources [16,17].

Cd accumulates most extensively in the proximal tubular epithelial cells of the kidneys, and the kidney burden of Cd as μg/ g tissue weight increases with age [18,19]. Approximately 0.001–0.005% of Cd in the body is excreted in the urine each day, and the biological

half-life of Cd in the kidney cortex is estimated to be 30 years for non-smokers [19,20]. In Japanese residents of a Cd pollution area, the average half-life of the metal among those with a lower body burden (urinary Cd < 5 µg/L) was 23.4 years; in those with a higher body burden (urinary Cd > 5 µg/L), the average half-life was 12.4 years [21,22]. Thus, the lower the body burden, the longer the half-life of Cd.

To safeguard against excessive dietary Cd exposure, health guidance values have been established by the Joint FAO/WHO Expert Committee on Food Additives and Contaminants (JECFA) [23]. Based on a risk assessment model that assumed an increase in the excretion of β_2-microglobulin (β_2M, $E_{\beta2M}$) above 300 µg/g creatinine to be the "critical" toxicity endpoint, the tolerable intake level of Cd was set at 0.83 µg/kg body weight/day, and a urinary Cd excretion of 5.24 µg/g creatinine was considered to be the toxicity threshold level [23]. However, $E_{\beta2M}$ levels of 100–299, 300–999, and $\geq$1000 µg/g creatinine were found to be associated with 4.7-, 6.2- and 10.5-fold increases in the risk of an estimated GFR (eGFR) $\leq$ 60 mL/min/1.73 m^2, commensurate with chronic kidney disease (CKD) [24]. Thus, $E_{\beta2M}$ above 300 µg/g creatinine does not appear to be an early warning sign of the nephrotoxicity of Cd. The utility of β_2M excretion as a toxicity criterion to derive Cd health guidance values is questionable.

Presently, the benchmark dose (BMD) has often been used to derive health guidance values instead of the no-observed-adverse-effect level (NOAEL) [25–27]. The BMD is a dose level, derived from an estimated dose–response curve, associated with a specified change in response, termed benchmark response (BMR) which can be set at 1%, 5%, and 10% as required. BMD rectifies some of the shortcomings of the NOAEL [28,29]. For a continuous endpoint, the lower 95% confidence bound of BMD, termed BMDL value derived at 5% BMR has been viewed as NOAEL equivalent or the level below which adverse health effect could be negligible [25].

The present study had three aims. The first aim was to evaluate the validity of current kidney toxicity threshold level of E_{Cd} at 5.24 µg/g creatinine. The second aim was to define the BMDL values (NOAEL equivalents) of E_{Cd} from three indicators of renal effects; injury to kidney tubular epithelial cells, a defective tubular reabsorption of β_2M, and eGFR decline. Kidney tubular cell injury was indicated by an increase in excretion of N-acetyl-β-D-glucosaminidase (NAG, E_{NAG}). We postulated that the BMDL value of E_{Cd} for the E_{NAG} endpoint was the lowest, given that the E_{NAG} represents an early sign of the cytotoxicity of Cd. The third aim was to demonstrate an impact of methods used to normalize Cd, NAG and β_2M excretion rates. Accordingly, we assessed the validity of BMDL values of E_{Cd} obtained from normalizing excretion rates of Cd, NAG and β_2M to creatinine clearance (C_{cr}) as E_x/C_{cr} and to the excretion of creatinine (E_{cr}) as E_x/E_{cr}, where x = Cd, NAG or β_2M.

2. Materials and Methods

2.1. Study Subjects

We assembled archived data from 289 men and 445 women who were drawn from Bangkok (a low-exposure area) and from Cd contaminated areas of Mae Sot District (a high-exposure area) in Thailand [30]. The Ethical Committee of Chulalongkorn University and the Mae Sot Hospital Ethical Committee approved the study protocol. At the time of recruitment, all participants had lived at their current addresses for at least 30 years, and all gave informed consent prior to participation. Exclusion criteria were pregnancy, breastfeeding, a history of metalwork, and a hospital record or physician's diagnosis of an advanced chronic disease. Smoking, diabetes, hypertension, regular use of medications, educational level, occupational and family health history were ascertained by a 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, and/or a physician's diagnosis and prescription of anti-hypertensive medications.

2.2. Blood and Urine Sampling and Analyses

Simultaneous blood and urine sampling is required to normalize E_{Cd}, $E_{\beta 2M}$ and E_{NAG} to C_{cr} Accordingly, second morning urine samples were collected after an overnight fast, and whole blood samples were obtained within 3 h after the urine sampling. Aliquots of urine, whole blood and plasma were stored at $-20\,^\circ$C or $-80\,^\circ$C for later analysis. The assay for urine and plasma concentrations of creatinine ($[cr]_u$ and $[cr]_p$) was based on the Jaffe reaction. The urinary NAG assay was based on colorimetry (NAG test kit, Shionogi Pharmaceuticals, Sapporo, Japan). The urinary β_2M assay was based on the latex immunoagglutination method (LX test, Eiken 2MGII; Eiken and Shionogi Co., Tokyo, 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 it had the high sensitivity required to measure very low Cd concentrations. Multi-element standards (EM Science, EM Industries, Inc., Newark, NJ, USA) were used to calibrate the Cd analyses. The accuracy and precision of those analyses were ascertained with reference urine (Lyphochek®, Bio-Rad, Sydney, Australia). The low limit of detection (LOD) of urine Cd, calculated as 3 times the standard deviation of blank measurements was 0.05 µg/L. The Cd concentration assigned to samples with Cd below the detection limit was the detection limit divided by the square root of 2 [31].

Based on E_{Cd} data reported for Mae Sot residents that could be reliably quantified by atomic absorption spectrophotometry (AAS), the $[Cd]_u$ in samples from Mae Sot group was determined by AAS (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. The LOD of Cd quantitation, calculated as 3 times the standard deviation of blank measurements was 0.06 µg/L. None of the urine samples from this group was found to have a $[Cd]_u$ below the detection limit.

2.3. Estimated Glomerular Filtration Rate (eGFR)

The GFR is the product of nephron number and mean single nephron GFR, and in theory the GFR is indicative of nephron function [32–34]. In practice, the GFR is estimated from established chronic kidney disease-epidemiology collaboration (CKD-EPI) equations, and is reported as eGFR.

Male eGFR = $141 \times$ [serum creatinine/0.9]$^y \times 0.993^{age}$, where y = -0.411 if serum creatinine ≤ 0.9 mg/dL or -1.209 if serum creatinine > 0.9 mg/dL.

Female eGFR = $144 \times$ [serum creatinine/0.7]$^y \times 0.993^{age}$, where y = -0.329 if serum creatinine ≤ 0.7 mg/dL or -1.209 if serum creatinine > 0.7 mg/dL.

These CKD-EPI equations have been validated with inulin clearance, and they are considered to be the most accurate eGFR calculating equations [35].

2.4. Normalization of E_{Cd}, $E_{\beta 2M}$ and E_{NAG} to E_{cr} and C_{cr}

E_x was normalized to E_{cr} as $[x]_u/[cr]_u$, where x = Cd, β_2M or NAG; $[x]_u$ = urine concentration of x (mass/volume); and $[cr]_u$ = urine creatinine concentration (mg/dL). The ratio $[x]_u/[cr]_u$ was expressed in µg/g of creatinine.

E_x was normalized to C_{cr} as $E_x/C_{cr} = [x]_u[cr]_p/[cr]_u$, where x = Cd, β_2M or NAG; $[x]_u$ = urine concentration of x (mass/volume); $[cr]_p$ = plasma creatinine concentration (mg/dL); and $[cr]_u$ = urine creatinine concentration (mg/dL). E_x/C_{cr} was expressed as the excretion of x per volume of filtrate [36]. Derivation of an equation for C_{cr} normalization is in Appendix A. Demonstration that E_{Cd}/C_{cr} is unaffected by muscle mass is in Appendix B.

2.5. Benchmark Dose Computation and Benchmark Response (BMR) Setting

We used the web-based PROAST software version 70.1 (https://proastweb.rivm.nl (accessed on 9 June 2022) to compute the BMD figures for Cd exposure as E_{Cd}/E_{cr} or E_{Cd}/C_{cr} associated with three effect markers, eGFR, E_{NAG}, and $E_{\beta 2M}$. A specific effect size, termed BMR, was set at 5% for all continuous endpoints, given that the BMDL values obtained could serve as the point of departure to derive health guidance values [25–27]. BMD values

were computed from fitting datasets to multiple dose–response models such as inverse exponential, natural logarithmic, exponential, and Hill models. BMDL/BMDU values were computed from those showing a statistically significant dose–response relationship. Model averaging was used instead of a single model analysis to account for model uncertainty.

The BMR was set at a 10% increase in the prevalence of the following quantal endpoints; eGFR ≤ 60 mL/min/1.73 m^2, $E_{NAG}/E_{cr} \geq 4$ U/g creatinine, $E_{\beta 2MG}/E_{cr} \geq 300$ μg/g creatinine, $E_{NAG}/C_{cr} \times 100 \geq 4$ U/L filtrate, and $E_{\beta 2MG}/C_{cr} \times 100 \geq 300$ μg/L filtrate. These endpoints were indicative of the presence of CKD, toxic injury to kidney tubular epithelial cells, and a reduction in tubular reabsorption of filtered protein, respectively. BMD values were calculated from fitting datasets to multiple dose–response models that included two-stage, logarithmic logistic, Weibull, logarithmic probability, gamma, exponential and Hill models.

The BMDL and BMDU corresponded to the lower bound and upper bound of the 95% confidence interval (CI) of BMD. The BMDL/BMDU were from model averaging using bootstrap with 200 repeats. A wider BMDL-BMDU difference, a higher statistical uncertainty in the dataset [28,29,37].

2.6. Statistical Analysis

Data were analyzed with IBM SPSS Statistics 21 (IBM Inc., New York, NY, USA). The distributions of eGFR and the excretion of Cd, β$_2$M and NAG were examined for skewness, and those showing rightward skewing were subjected to logarithmic transformation before analysis. The departure of a given variable from a normal distribution was assessed with the one-sample Kolmogorov–Smirnov test. The Kruskal–Wallis test was used to compare mean differences across three eGFR groups. 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. For each analysis, *p*-values ≤ 0.05 for two-tailed tests were assumed to indicate statistical significance.

3. Results

3.1. Characteristics of Males and Females of Low- and High-Exposure Locations

Among 734 participants, 100 males and 100 females were residents of a low-exposure area (Bangkok), and 189 males and 345 females were residents of a high-exposure area (Mae Sot) residents (Table 1).

The overall mean age of 734 participants was 48.1 years. Of the total participants, 42.8% were smokers to include those who had quite less than 10 years. Another 31.7% of participants had hypertension and only 1.5% had diabetes. The overall % of CKD was 9%. None of subjects of the low-exposure group had CKD, while the % of CKD among males and females of the high-exposure group were 15.3% and 10.7%, respectively.

The overall % of subjects with a sign of tubular cell injury evident from $E_{NAG}/E_{cr} \geq 4$ U/g creatinine was 76.2%, while the overall % of subjects showing a sign of tubular dysfunction was 39.8%.

Smoking was particularly high among males in the Mae Sot group (81.5%) than in males of the Bangkok group (47%). All females of the Bangkok group were non-smokers, while 32.8% of female residents of Mae Sot smoked cigarettes.

Mean E_{Cd} for smokers in Mae Sot was 18.3-fold higher than that of smokers in Bangkok (5.87 vs. 0.32 μg/L, *p* < 0.001). The mean E_{Cd} was 24.9-fold higher in non-smokers of the Mae Sot than non-smokers of Bangkok (5.22 vs. 0.21 μg/L, *p* < 0.001).

For the Bangkok group, mean [cr]$_p$ was lower in females than males (*p* < 0.001) as was mean E_{NAG}/C_{cr} (*p* = 0.023). For the Mae Sot group, mean [cr]$_p$ and mean [cr]$_u$ in females were lower than males (*p* < 0.001 for both parameters) as were mean [Cd]$_u$ (*p* = 0.020) and mean E_{Cd}/C_{cr} (*p* = 0.021). In contrast, mean E_{NAG}/E_{cr} in females was higher than that of males (*p* < 0.001).

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Table 1. Characteristics of the study subjects, stratified by residential location and sex.

Parameters	All Subjects n 734	Low Exposure (Bangkok)		High Exposure (Mae Sot)	
		Males, n 100	Females, n 100	Males, n 189	Females, n 345
Smoking (%)	42.8	47	0 ***	81.5	32.8 ***
Hypertension (%)	31.7	27	12 **	30.2	39.7 *
Diabetes (%)	1.5	0	0	3.2	1.4
Age, years	48.1 ± 11.0	36.2 ± 10	42.3 ± 9.5 ***	53.3 ± 11.2	50.4 ± 8.2 *
BMI, kg/m^2	23.2 ± 3.8	23.2 ± 3.5	23.1 ± 3.8	22.0 ± 3.2	23.8 ± 4.1 ***
eGFR [a], mL/min/1.73 m^2	91.0 ± 21.7	104 ± 17	106 ± 14	83 ± 23	87 ± 21 *
Low eGFR [b] (%)	9	0	0	15.3	10.7
Plasma creatinine, mg/dL	0.85 (0.74)	0.92 (0.73)	0.67 (0.93) ***	1.04 (1.02)	0.79 (0.90) ***
Urine creatinine, mg/dL	85.59 (1.54)	62.27 (1.68)	48.56 (1.70)	122.50 (1.40)	90.90 (1.50) ***
Urine NAG, U/L	5.48 (1.81)	2.11 (1.93)	1.95 (1.91)	7.83 (1.65)	8.02 (1.81)
Urine β_2M, μg/L	112.72 (3.10)	4.95 (3.70)	3.52 (3.39)	473.79 (2.88)	340.18 (2.63)
Urine Cd, μg/L	2.34 (2.32)	0.24 (2.83)	0.22 (2.84)	6.34 (2.08)	5.14 (1.92) *
Normalized to E$_{cr}$ as E$_x$/E$_{cr}$ [c]					
E$_{NAG}$/E$_{cr}$, μg/g creatinine	6.40 (1.56)	3.38 (1.73)	4.01 (1.56)	6.39 (1.42)	8.83 (1.57) ***
E$_{\beta 2M}$/E$_{cr}$, U/g creatinine	130.52 (2.97)	7.96 (3.51)	7.26 (3.47)	387.74 (2.48)	374.25 (2.62)
E$_{Cd}$/E$_{cr}$, μg/g creatinine	2.73 (2.09)	0.39 (2.58)	0.46 (2.47)	5.18 (1.83)	5.66 (1.82)
E$_{NAG}$/E$_{cr}$ ≥ 4 U/g creatinine (%)	76.2	48.0	60.0	79.4	87.2 *
E$_{\beta 2M}$/E$_{cr}$ ≥ 300 μg/g creatinine (%)	39.8	7.0	1.0 *	47.6	46.4
Normalized to C$_{cr}$ as E$_x$/C$_{cr}$ [d]					
E$_{NAG}$/C$_{cr}$ × 100, U/L filtrate	5.42 (1.59)	3.12 (1.70)	2.69 (1.71) *	6.64 (1.50)	6.98 (1.56)
E$_{\beta 2M}$/C$_{cr}$ × 100, μg/L filtrate	110.5 (15.3)	7.34 (3.48)	4.86 (3.55)	402.82 (2.91)	295.87 (2.63)
E$_{Cd}$/C$_{cr}$ × 100, μg/L filtrate	2.31 (2.15)	0.36 (2.53)	0.31 (2.61)	5.38 (2.01)	4.47 (1.83) *
E$_{NAG}$/C$_{cr}$ × 100 ≥ 4 U/L filtrate (%)	66.2	41.0	21.0 *	81.5	78.3
E$_{\beta 2M}$/C$_{cr}$ × 100 ≥ 300 μg/L filtrate (%)	37.1	7.0	0 **	51.3	48.7

n, number of subjects; BMI, body mass index; eGFR, estimated glomerular filtration rate; E$_x$, excretion of x; cr, creatinine; C$_{cr}$, creatinine clearance; β_2M, β_2-microglobulin; NAG, N-acetyl-β-D-glucosaminidase, Cd, cadmium; [a] eGFR, was determined by Chronic Kidney Disease Epidemiology Collaboration equations [33]. [b] Low eGFR was defined as eGFR ≤ 60 mL/min/1.73 m^2. [c] E$_x$/E$_{cr}$ = [x]$_u$/[cr]$_u$; [d] E$_x$/C$_{cr}$ = [x]$_u$[cr]$_p$/[cr]$_u$, where x = Cd, β_2M or NAG [36]. Data for age, eGFR and BMI are arithmetic means ± standard deviation (SD). Data for all other continuous variables are geometric mean (geometric standard deviation) values. Data for BMI values are from 709 subjects; data for all other variables are from 734 subjects. For each test, $p ≤ 0.05$ identifies statistical significance, determined by Pearson's Chi-square test for percentage differences and the Mann–Whitney U-test for mean differences between men or women of low- vs. high-exposure locations. * $p = 0.010$–0.035; ** $p = 0.007$; *** $p < 0.001$.

3.2. Characteristics of Study Subjects, Stratified by eGFR Values

In current practice, staging of CKD is based on eGFR values where CKD stages 1, 2, 3a, 3b, 4, and 5 correspond to eGFR of 90–119, 60–89, 45–59, 30–44, 15–29, and <15 mL/min/1.73 m^2, respectively [33]. For dichotomous comparisons, CKD is defined as eGFR ≤ 60 mL/min/1.73 m^2 [33].

Table 2 provides data from 734 participants stratified by eGFR levels 1, 2 and 3 corresponding to eGFR ≥ 90, 61–89, and ≤ 60 mL/min/1.73 m^2, respectively.

The percentages of females and those with hypertension in each eGFR group did not differ, while % of diabetes (6.1%) and smoking (63.6%) were the highest in the lowest eGFR group. None of subjects from a low exposure area had low eGFR. Subjects of the low eGFR group was the oldest (mean age 63.2), who also had the lowest mean BMI [($p = 0.020$). Mean [cr]$_p$, mean [cr]$_u$ and mean [Cd]$_u$ all were the highest in the eGFR level 3, middle in eGFR level 2, and the lowest in eGFR level 3 as were the means for E$_{cr}$- and C$_{cr}$-normalized E$_{Cd}$, E$_{\beta 2MG}$, E$_{NAG}$.

3.3. BMDL and BMDU Values of Cadmium Excretion from E$_{cr}$-Normalized Dataset

The BMDL and BMDU values of E$_{Cd}$/E$_{cr}$ computed from three kidney toxicity endpoints; tubular cell injury, tubular dysfunction and eGFR reduction are provided separately for males and females (Table 3).

Table 2. Characteristics of the study subjects stratified by eGFR values.

Parameters	eGFR, mL/min/1.73 m^2			
	Level 1, *n* 410	Level 2, *n* 258	Level 3, *n* 66	*p*
Low-exposure area (%)	40.5	13.2	0	<0.001
Females (%)	59.8	63.2	56.1	0.494
Smoking (%)	34.6	50.4	63.6	<0.001
Diabetes (%)	0	2.7	6.1	<0.001
Hypertension (%)	29.5	32.9	40.1	0.159
Age, years	43.0 ± 9.0	52.2 ± 8.3	63.2 ± 11.7	<0.001
BMI, kg/m^2	23.5 ± 3.7	23.0 ± 3.9	22.0 ± 4.2	0.020
eGFR [a], mL/min/1.73 m^2	106.8 ± 9.5	77.2 ± 8.0	46.1 ± 11.0	<0.001
Plasma creatinine, mg/dL	0.73 (0.87)	0.95 (0.75)	1.42 (1.34)	<0.001
Urine creatinine, mg/dL	73.60 (1.60)	102.3 (1.46)	108.4 (1.41)	<0.001
Urine NAG, U/L	4.68 (1.85)	6.22 (1.74)	8.97(1.77)	<0.001
Urine β$_2$M, μg/L	50.26 (3.14)	165.43 (2.82)	3439.16 (3.63)	<0.001
Urine Cd, μg/L	1.26 (2.43)	4.40 (2.09)	9.39 (2.30)	<0.001
Normalized to E$_{cr}$ as E$_x$/E$_{cr}$ [b]				
E$_{NAG}$/E$_{cr}$, U/g creatinine	6.35 (1.57)	6.08 (1.52)	8.27 (1.68)	0.049
E$_{β2M}$/E$_{cr}$, μg/g creatinine	68.24 (2.98)	161.69 (2.73)	3172.49 (3.58)	<0.001
E$_{Cd}$/E$_{cr}$, μg/g creatinine	1.70 (2.18)	4.30 (1.92)	8.66 (2.12)	<0.001
Normalized to C$_{cr}$ as E$_x$/C$_{cr}$ [c]				
E$_{NAG}$/C$_{cr}$ × 100, U/L filtrate	4.61 (1.56)	5.76 (1.50)	11.73 (1.93)	<0.001
E$_{β2M}$/C$_{cr}$ × 100, μg/L filtrate	49.58 (2.99)	153.15 (2.74)	4497.44 (3.78)	<0.001
E$_{Cd}$/C$_{cr}$ × 100, μg/L filtrate	1.24 (2.20)	4.08 (1.96)	12.28 (2.44)	<0.001

n, number of subjects; BMI, body mass index; eGFR, estimated glomerular filtration rate; E$_x$, excretion of x; cr, creatinine; C$_{cr}$, creatinine clearance; β$_2$M, β$_2$-microglobulin; NAG, N-acetyl-β-D-glucosaminidase, Cd, cadmium; [a] eGFR, was determined by Chronic Kidney Disease Epidemiology Collaboration (CKD–EPI) equations [33] and eGFR levels 1, 2 and 3 corresponded to eGFR ≥ 90, 61–89, and ≤ 60 mL/min/1.73 m^2, respectively. [b] E$_x$/E$_{cr}$ = [x]$_u$/[cr]$_u$; [c] E$_x$/C$_{cr}$ = [x]$_u$[cr]$_p$/[cr]$_u$, where x = Cd, β$_2$M or NAG [36]. Data for age, eGFR and BMI are arithmetic means ± standard deviation (SD). Data for all other continuous variables are geometric mean (geometric standard deviation) values. Data for BMI are from 709 subjects; data for all other variables are from 734 subjects. For each test, *p* ≤ 0.05 identifies statistical significance, determined by Pearson Chi-Square test for % differences and by Kruskal–Wallis test for mean differences across three eGFR groups.

Table 3. BMDL and BMDU values of cadmium accumulation associated with adverse effects on kidneys according to E$_{cr}$-normalized dataset.

Endpoints	Males			Females		
	BMDL	BMDU	U/L Ratio	BMDL	BMDU	U/L Ratio
Continuous endpoints						
5% Increase of E$_{NAG}$/E$_{cr}$	0.060	0.504	8.44	0.069	0.537	7.79
5% Increase of E$_{β2MG}$/E$_{cr}$	0.019	0.411	22	0.022	0.491	23
5% Decrease of eGFR	1.57×10^{-6}	0.427	2.7×10^5	1.88×10^{-5}	0.495	2.6×10^4
Quantal endpoints						
10% Increase in prevalence of tubular cell injury [a]	1.48×10^{-3}	0.560	378	3.17×10^{-4}	0.706	2227
10% Increase in prevalence of defective tubular reabsorption [b]	0.469	0.973	2.1	0.733	1.29	1.8
10% Increase in prevalence of CKD [c]	3.260	7.460	2.29	4.980	9.680	1.94

BMDL, and BMDU values of E$_{Cd}$/E$_{cr}$ were as μg/g creatinine. CI, confidence interval; U/L, BMDU/BMDL ratio. U/L ≥ 100 indicated a high degree of statistical uncertainty. [a] E$_{NAG}$/E$_{cr}$ ≥ 4 U/g creatinine reflected tubular cell injury. [b] E$_{β2MG}$/E$_{cr}$ ≥ 300 μg/g creatinine reflected a defective tubular reabsorption. [c] CKD was defined as eGFR ≤ 60 mL/min/1.73 m^2.

In continuous dose–response analyses, the respective BMDL/BMDU of E$_{Cd}$/E$_{cr}$ associated with a tubular cell injury in men and women were 0.060/0.504 and 0.069/0.537 μg/g creatinine (Figure S1), while the corresponding BMDL/BMDU of E$_{Cd}$/E$_{cr}$ associated with a tubular dysfunction were 0.019/0.411 and 0.022/0.491 μg/g creatinine (Figure S2). The BMDL (NOAEL equivalent) values of E$_{Cd}$/E$_{cr}$ associated with these tubular toxicity indicators could be reliably determined, given that the BMDU/BMDL ratios ranged between

8.44 and 23. In contrast, the BMDU/BMDL ratios of E_{Cd}/E_{cr} associated with a 5% decrease in eGFR were extremely large (Figure S3). Thus, the respective BMDL/BMDU values of E_{Cd}/E_{cr} associated with eGFR effect in men and women of $1.57 \times 10^{-6}/0.427$ and $1.88 \times 10^{-5}/0.495$ µg/g creatinine were not reliably determined.

In quantal dose–response analyses (Figure S4), the respective BMDU/BMDL ratios of E_{Cd}/E_{cr} associated with a 10% increase in the prevalence of tubular cell injury in men and women were $1.48 \times 10^{-3}/0.56$ and $3.17 \times 10^{-4}/0.706$ µg/g creatinine. These BMDL values were not reliable due to the extremely large BMDL/BMDU ratios of E_{Cd}/E_{cr} in both men and women. The respective BMDL/BMDU values of E_{Cd}/E_{cr} associated with a 10% incidence of an increase in prevalence of tubular dysfunction were reliably determined as 0.469/0.973 and 0.733/1.29 µg/g creatinine. Likewise, reliable BMDL/BMDU values of E_{Cd}/E_{cr} associated with a 10% increase in the prevalence of CKD in men and women were 3.26/7.46 and 4.98/9.68 µg/g creatinine.

3.4. BMDL and BMDU Valuse of Cadmium Excretion from C_{cr}-Normalized Dataset

The BMDL and BMDU values of E_{Cd}/C_{cr} computed from three kidney toxicity endpoints; tubular cell injury, tubular dysfunction and eGFR reduction are provided separately for males and females (Table 4).

In continuous dose–response analyses, the respective BMDL/BMDU of $E_{Cd}/C_{cr} \times 100$ associated with a tubular cell injury in men and women were 0.067/0.394, and 0.067/0.399 µg/L filtrate (Figure S5), while the corresponding BMDL/BMDU of $E_{Cd}/C_{cr} \times 100$ associated with a 5% reduction in reabsorption of β_2M were $0.41 \times 10^{-4}/0.038$, and $0.16 \times 10^{-3}/0.040$ µg/L (Figure S6). The BMDL (NOAEL equivalent) values of $E_{Cd}/C_{cr} \times 100$ associated with a defective tubular reabsorption in men and women could not be reliably determined, due extremely large BMDU/BMDL ratios, indicative of a high degree of statistical uncertainty. In contrast, the BMDU/BMDL values of $E_{Cd}/C_{cr} \times 100$ associated with a 5% eGFR decline were determined with a sufficiently high degree of statistical certainty as 0.080/0.714 and 0.098/0.812 µg/L filtrate in men and women, respectively (Figure S7).

Table 4. BMDL and BMDU values of cadmium excretion levels associated with adverse effects on kidneys according to C_{cr}-normalized datasets.

Endpoints	Males			Females		
	BMDL	BMDU	U/L Ratio	BMDL	BMDU	U/L Ratio
Continuous endpoints						
5% Increase of $E_{NAG}/C_{cr} \times 100$	0.067	0.394	5.88	0.067	0.399	5.96
5% Increase of $E_{\beta2MG}/C_{cr} \times 100$	0.41×10^{-4}	0.38	929	0.16×10^{-3}	0.040	255
5% Decrease of eGFR	0.080	0.714	8.92	0.098	0.812	8.29
Quantal endpoints						
10% Increase in prevalence of tubular cell injury [a]	0.254	0.458	1.80	0.366	0.614	1.68
10% Increase in prevalence of tubular dysfunction [b]	0.456	0.807	1.77	0.500	0.805	1.61
10% Increase in prevalence of CKD [c]	5.060	8.660	1.71	5.150	7.620	1.48

BMDL and BMDU values of $E_{Cd}/C_{cr} \times 100$ were in µg/L filtrate. CI, confidence interval; U/L, BMDU/BMDL ratio. U/L $\geq$ 100 indicated a high degree of statistical uncertainty. [a] $E_{NAG}/C_{cr} \times 100 \geq 4$ U/L filtrate reflected tubular cell injury. [b] $E_{\beta2MG}/C_{cr} \times 100 \geq 300$ µg/L filtrate reflected a defective tubular reabsorption. [c] CKD was defined as eGFR $\leq$ 60 mL/min/1.73 m^2.

In quantal dose–response analyses (Figure S8), the respective BMDU/BMDL ratios of E_{Cd}/C_{cr} associated with a 10% increase in the prevalence of tubular cell injury in men and women 2.54/4.58, and 3.66/6.14 µg/L filtrate, respectively. The respective BMDL/BMDU values of $E_{Cd}/C_{cr} \times 100$ associated with a 10% incidence of an increase in prevalence of defective tubular dysfunction were 0.456/0.807 and 0.5/0.805 µg/ L filtrate, respectively. Likewise, reliable BMDL/BMDU values of $E_{Cd}/C_{cr} \times 100$ associated with a 10% increase

in the prevalence of CKD in men and women were 5.06/8.66 µg/L filtrate in men and 5.15/7.62 µg/L filtrate in women.

4. Discussion

To the best of our knowledge, we herein provide, for the first time, BMDL values of Cd excretion levels that are derived simultaneously from three toxicity endpoints; injury to kidney tubular epithelial cells, a defective tubular reabsorption of β_2MG, and eGFR decline. As age distribution histogram indicates (Figure S9), these BMDL values of permissible Cd accumulation levels can be generalized to an entire adult population. A Cd accumulation level producing E_{Cd}/C_{cr} of 0.67 ng/L filtrate in men and women could be considered as Cd accumulation levels below which renal effects are likely to be negligible (Figure S5). A reduction in eGFR may follow when Cd accumulation levels rise to 0.80 ng/L filtrate in men and 0.98 ng/L filtrate in women (Figure S7).

The reported BMD values of E_{Cd}/E_{cr} associated with adverse effects on kidneys vary among studies. The variability was most likely due to differences in age of subjects included in an analysis as well as differences in the dose–response models applied to the datasets [25–27]. It is noteworthy that most BMD values of E_{Cd} associated with adverse kidney effects were derived from those age 50 years or older. Consequently, most BMD figures previously published were not generalizable to the entire adult population. For a quantal endpoint, different cut-off values constituted an additional source of variable BMD values. There is a need to standardize the BMD approach to advance Cd toxicological risk assessment. Age should form an important consideration in deriving BMDL.

In the present study, we used E_{NAG}/E_{cr} of 4 U/g creatinine as the cut-off value to reflect injury to kidney tubular epithelial cells (Figure S1). E_{NAG} is considered to be proportional to the number of surviving nephrons because NAG in urine originated from injured or dying tubular cells [38–40]. In a United Kingdom (U.K.) study, a dose–response relationship was observed between E_{Cd} and E_{NAG}; E_{Cd}/E_{cr} of 0.5 µg/g creatinine was associated with 2.6- and 3.6-fold increases in the prevalence of $E_{NAG}/E_{cr} > 2$ U/g creatinine, as compared with a E_{Cd}/E_{cr} of 0.3 and < 0.5 µg/g creatinine, respectively [41]. The BMDL/BMDU figures derived by us from E_{NAG} endpoint were in line with the U.K. study. Apparently, these NOAEL equivalents of E_{Cd}/E_{cr} associated with tubular epithelial cell injury were lower than E_{Cd}/E_{cr} of 5.24 µg/g creatinine, suggested to be a nephrotoxicity threshold level of Cd [18].

The BMDL values of E_{Cd}/E_{cr} derived from kidney tubular cell injury endpoint were 0.060 and 0.069 µg/g creatinine in men and women, respectively (Figure S1). The respective BMDL values of E_{Cd}/E_{cr} associated with a 5% decrease of tubular reabsorption of β_2M in men and women are 0.019 and 0.022 µg/g creatinine (Figure S2). In contrast, the BMDL values of E_{Cd}/E_{cr} associated with a 5% eGFR decline could not be reliably determined (U/L ratios of 10^4–10^5). These findings were consistent with data from a conventional dose–response analysis, where an inverse relationship between E_{Cd} and eGFR became statistically significant only when E_{Cd} was normalized to C_{cr} [42].

Because of high variance in datasets introduced by E_{cr}-normalization, the effect of Cd on eGFR was not realized. In a systematic review and meta-analysis of pooled data from 28 studies, the risk of proteinuria was increased by 1.35-fold when comparing the highest vs. lowest category of Cd dose metrics, but an increase in the risk of low eGFR was statistically insignificant ($p = 0.10$) [43]. An erroneous conclusion that chronic Cd exposure was not associated with a progressive eGFR reduction was also made in another systematic review [44].

In a meta-analysis of pooled data from 30 publications, the BMD of E_{Cd}/E_{cr} for tubular injury (E_{NAG}) was 1.67 µg/g creatinine [45]. In another study using data from 469 men and 465 women who were resident of Zhejiang Province, China, the E_{Cd}/E_{cr} BMD/BMDL figures based on $E_{\beta2M}$ and E_{NAG} were 1.24/0.62 and 0.85/0.49 µg/g creatinine in men, respectively [46]. The corresponding figures for women were 1.35/0.64 and 1.36/0.65 µg/g

creatinine, respectively. Of note, in men only, E_{Cd}/E_{cr} BMD/BMDL figures for $E_{\beta 2MG}$ were higher than those for E_{NAG}. This was seen in a Swedish study [47] and our datasets.

In a study of data from 410 men and 418 women, aged 40–59 years, who were residents of non-pollution areas of Japan, the E_{Cd}/E_{cr} BMD figures for $E_{\beta 2M}$ endpoint were 0.6–1.2 and 0.6–2.3 µg/g creatinine in men and women, respectively [48].

According to a quantal endpoint of $E_{\beta 2M}/E_{cr} \geq 300$ µg/g creatinine, the BMDL value E_{Cd}/E_{cr} values were 0.469 and 0.733 µg/g creatinine in men and women, respectively (Figure S4). These data imply that the prevalence of Cd-induced tubular dysfunction was likely to be smaller than 10% at E_{Cd}/E_{cr} 0.469 µg/g creatinine in men, and 0.733 µg/g creatinine in women. Of concern, these E_{Cd}/E_{cr} levels have been associated with increased risk of CKD in the general populations in the U.S. [49] Spain [50], Taiwan [51] and China [52,53]. Arguably, $E_{\beta 2M}$ is not a sensitive toxicity endpoint and consequently the dietary exposure guideline of tolerable intake at 0.83 µg/kg body weight/day derived from $E_{\beta 2M}/E_{cr} \geq 300$ µg/g creatinine endpoint does not afford sufficient health protection.

The BMD values for E_{NAG} and eGFR reduction in a study of 790 Swedish women, aged 53–64 years, were 0.5–0.8 and 0.7–1.2 µg/g creatinine, respectively [47]. A Cd-induced kidney tubular cell injury and eGFR reduction could be more suitable endpoints for health risk calculation than $E_{\beta 2M}$. In clinical trials, the successful treatment of kidney disease is judged by the attenuation of a decline in eGFR [33,54]. CKD is a progressive syndrome with high morbidity and mortality, and affects 8% to 16% of the world's population. The continuing rise in the incidence of CKD globally is a cause of concern [54].

It has long been viewed that excreted Cd included Cd molecules that pass through the glomerular filtration membrane into the filtrate but are not reabsorbed. However, it is noteworthy that the excreted Cd originates from injured or dying tubular cells [40]. In an analogy to E_{NAG}, Cd excretion is a manifestation of the cytotoxicity of its accumulation in kidneys' tubular cells. In a histopathological examination of kidney biopsies from healthy kidney transplant donors [55], the degree of tubular atrophy was positively associated with the level of Cd accumulation. Tubular atrophy was observed at relatively low Cd levels [55].

The reductions in eGFR due to Cd nephropathy have often been attributed to glomerular injury. However, current evidence suggests that sufficient tubular injury disables glomerular filtration and leads to nephron atrophy and GFR loss [40,56]. Cd inflicts tubular cell injury at low intracellular concentrations, and the toxicity intensifies as Cd concentration rises [40]. The Cd-induced tubular injury disables glomerular filtration, leading to nephron atrophy, glomerulosclerosis, and interstitial inflammation and fibrosis. A reduction in tubular reabsorption of filtered proteins, retinal binding protein (RBP) and $\beta_2 MG$ follows tubular atrophy and nephron loss. An increase of $\beta_2 M$ excretion to levels above 300 µg/g creatinine is speculatively due to effects of Cd on both tubular reabsorption and nephron number.

Figure 1 depicts the pathogenesis of Cd-induced nephropathy that originates from tubular injury deduced from the BMDL values of E_{Cd}/C_{cr} provided in Table 4.

In theory, an acceptable level of environmental exposure to Cd should be derived from the most sensitive toxicity endpoint, which is the one with the lowest BMDL value [27]. In the present study, BMDL for a 5% increase of $E_{\beta 2M}/C_{cr}$ could not be reliably determined (Table 4), thereby arguing that the utility of $E_{\beta 2M}$ as a toxicity criterion is not appropriate. The BMDL value of E_{Cd} for E_{NAG} endpoint appeared to be the lowest (Table 4). Thus, a tolerable intake level of Cd derived from E_{NAG}/C_{cr} endpoint will be sufficiently low to protect against Cd-induced toxic tubular cell injury. This E_{NAG}-based tolerable intake level of Cd will be lower than a tolerable intake level of 0.83 µg/kg body weight per day that was derived from $E_{\beta 2M}$ endpoint.

The protein $\beta_2 M$, with a molecular weight of 11,800 Da, is synthesized and shed by all nucleated cells in the body [57]. Owing to its small mass, $\beta_2 M$ is filtered freely by the glomeruli and is reabsorbed almost completely by the kidney's tubular epithelial cells [58–60]. Increased $\beta_2 M$ excretion has been used as an indicator of impaired tubular

re-absorptive function. It is noteworthy that β_2M production rises in response to many inflammatory and neoplastic conditions [61] and this compromises its utility as a marker of tubulopathy. If reabsorption rates of β_2M per nephron remain constant as its production rates change, excretion will vary directly with its production [Appendix C]. If the production and reabsorption per nephron remain constant as nephrons are lost, the excretion of β_2M will rise [Appendix D].
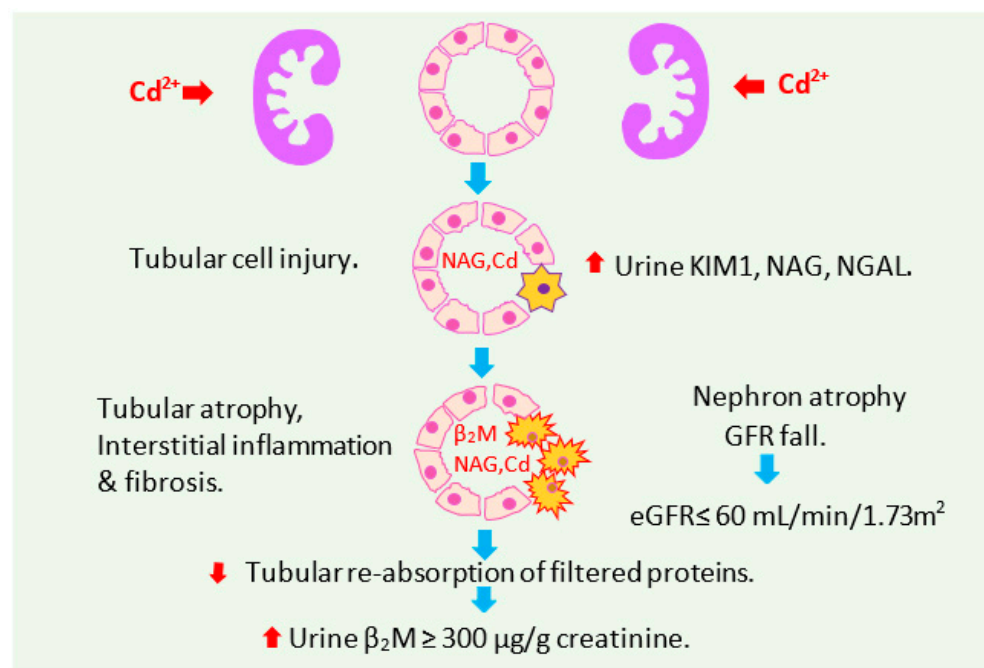
Figure 1. Sequential outcomes of tubular cell toxic injury of cadmium accumulation in kidneys. Cd inflicts tubular cell injury at low intracellular concentrations, and the toxicity intensifies as Cd concentration rises [40]. Tubular injury disables glomerular filtration, causing nephron atrophy, glomerulosclerosis, and interstitial inflammation and fibrosis [40,55,56]. A reduction in tubular reabsorption of filtered proteins, RBP and β_2MG follows tubular atrophy and a substantial nephron loss. Abbreviation: KIM1, kidney injury molecule 1; NAG, N-acetyl-β-D-glucosaminidase, NGAL, neutrophil gelatinase associated lipocalin; RBP, retinal binding protein; β_2M, β_2-microglobulin.

Consistent with the above notions are data in Table 2 where mean $E_{\beta2M}/C_{cr}$ in those with low eGFR was 90-fold higher than those with eGFR $\geq$ 90 mL/min/1.73 m^2, while there was only a 3-fold increase in $E_{\beta2M}/C_{cr}$ in those with moderate eGFR (eGFR 61–89 mL/min/1.73 m^2). $E_{\beta2M}$ is a function of nephron numbers and tubular reabsorption activity, a substantial increase in $E_{\beta2M}$ can be expected when nephrons are lost [24]. Accordingly, the sue of $E_{\beta2M}$ as a "critical" effect of Cd accumulation in kidney tubular cells is inappropriate.

5. Conclusions

The BMDL/BMDU values of E_{Cd}/C_{cr} associated with a 5% increase of kidney tubular cell injury are 0.67/3.94 and 0.67/3.99 ng/L filtrate in men and women, respectively. The respective BMDL/BMDU values of E_{Cd}/C_{cr} associated with a 5% eGFR decline in men and women are 0.80/7.14 and 0.98/8.12 ng/L filtrate. A Cd accumulation level resulting in Cd excretion at 0.67 ng/L filtrate in men and women could be viewed as NOAEL equivalent and is a maximally permissible Cd accumulation level. A reduction in eGFR and CKD may follow when Cd excretion levels exceed this maximal permissible accumulation level of 0.67 μg/L of filtrate. Health guidance values for Cd derived from these NOAEL equivalents of Cd excretion levels are more likely to be low enough to carry a negligible adverse effect on kidneys.

Appendix A

An equation for Normalizing Excretion of x (E_x) to Creatinine Clearance (C_{cr}) [40]

Let V_u = urine flow rate, units of volume/time;

E_x = urinary excretion rate of substance x, units of mass/time;

$[x]_u$ = urinary concentration of substance x, units of mass/volume;

E_{cr} = urinary excretion rate of creatinine, units of mass/time;

$[cr]_p$ = plasma concentration of creatinine, units of mass/volume;

$[cr]_u$ = urine concentration of creatinine, units of mass/volume;

C_{cr} = renal creatinine clearance (an approximation of GFR) = $E_{cr}/[cr]_p$, units of volume/time;

E_x/C_{cr} = amount of x excreted per volume of filtrate, units of mass/volume.

$E_x/C_{cr} = [x]_u V_u/([cr]_u V_u/[cr]_p)$; cancelling V_u and rearranging,

$E_x/C_{cr} = [x]_u[cr]_p/[cr]_u$.

Appendix B

E_{Cd}/C_{cr} Is Unaffected by Muscle Mass [40]

(a)   Let

(b) V_u = urine flow rate

(c) $E_{Cd} = [Cd]_u V_u$;

(d) $E_{cr} = [cr]_u V_u$; and

(e) $C_{cr} = E_{cr}/[cr]_p = [cr]_u V_u/[cr]_p$.

E_{cr} is directly related to muscle mass [62]. According to equation (d), at a given C_{cr}, E_{cr} and $[cr]_p$ rise or fall by the same factor.

If E_{Cd} is normalized to E_{cr}, then $E_{Cd}/E_{cr} = [Cd]_u V_u/[cr]_u V_u$. Since E_{cr} is directly related to muscle mass, E_{Cd}/E_{cr} is inversely related to muscle mass at any E_{Cd}. The same is true of $[Cd]_u/[cr]_u$ after cancellation of V_u in the numerator and denominator.

If E_{Cd} is normalized to C_{cr}, then E_{Cd}/C_{cr}, i.e., $[Cd]_u V_u/[cr]_u V_u/[cr]_p$, is unaffected by muscle mass because at a given $C_{cr} = (E_{cr}/[cr]_p)$, E_{cr} and $[cr]_p$ rise or fall by the same factor as muscle mass varies. This fact remains true after simplification of the complex fraction to yield $E_{Cd}/C_{cr} = [Cd]_u[cr]_p/[cr]_u$.

Appendix C

Demonstration that $E_{\beta2M}$ May Rise because of Increased Endogenous Production [40]

Let $I_{\beta2M}$ = influx of β_2M from endogenous sources into plasma;

$[\beta_2M]_p$ = plasma concentration of β_2M, mg/L;

$F_{\beta2M}$ = rate of glomerular filtration of β_2M, mg/d;

$E_{\beta2M}$ = urinary excretion rate of β_2M, mg/d;

$TD_{\beta2M}$ = rate of tubular degradation of β_2M, mg/d;

GFR = glomerular filtration rate, L/d.

Assume that $F_{\beta2M} = GFR[\beta_2M]_p = E_{\beta2M} + TD_{\beta2M}$.

Assume an equilibrium between $I\beta_2M$ and $F\beta_2M$, and assume stable GFR. If $I_{\beta2M}$ rises, so do $[\beta_2M]_p$ and $F_{\beta2M}$. If $TD_{\beta2M}$ remains stable, $E_{\beta2M}$ must rise if $F_{\beta2M}$ rises. Thus $E_{\beta2M}$ may rise even though $TD_{\beta2M}$ has not fallen.

Appendix D

Demonstration that $E_{\beta2M}$ Rises if GFR Falls [40]

From Appendix C, $F_{\beta2M} = GFR[\beta_2M]_p = E_{\beta2M} + TD_{\beta2M}$. Dividing the equation on the right by GFR,

$[\beta_2M]_p = E_{\beta2M}/GFR + TD_{\beta2M}/GFR$.

Assume that $I_{\beta2M}$ and thus $F_{\beta2M}$ remain stable. Since $F_{\beta2M} = GFR[\beta_2M]_p$, $[\beta_2M]_p$ must rise reciprocally if GFR falls. Assume that $TD_{\beta2M}/GFR$ remains stable as GFR falls. If GFR has fallen, $TD_{\beta2M}$ must also fall. Since $F_{\beta2M}$ is constant and $F_{\beta2M} = E_{\beta2M} + TD_{\beta2M}$, $E_{\beta2M}$ must rise.

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