Journal of Trace Elements in Medicine and Biology 54 (2019) 206–213

Contents lists available at ScienceDirect

Journal of Trace Elements in Medicine and Biology

journal homepage: www.elsevier.com/locate/jtemb

Epidemiology

Systematic evaluation of exposure to trace elements and minerals in patients with chronic kidney disease of uncertain etiology (CKDu) in Sri Lanka

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ARTICLE INFO

Keywords:
Chronic kidney disease
Sri Lanka
Cadmium
Arsenic
Trace elements
Minerals

ABSTRACT

Chronic kidney disease of uncertain etiology (CKDu) in areas in and around Sri Lanka's North Central Province has been identified as a major non-communicable disease due to its high prevalence and the burden on the public health system. Controversial evidence relating to the etiology and risk factors of CKDu has been reported. The most debated is the role of trace elements such as Cd and As in the pathogenesis of CKDu.

Urine and hair samples collected from CKDu patients and healthy controls were measured for the concentration of different elements including Cd and As. To assess the possible environmental exposures, drinking water and rice samples collected from the affected areas as well as unaffected areas in the country were analyzed. Transmission electronic microscopic analysis of renal biopsies from CKDu patients was also performed. Analysis of drinking water and rice samples indicated that the levels of all minerals and trace elements analyzed including Cd and As were within the levels recommended by World Health Organization and Sri Lanka drinking water guidelines and did not suggest any form of contamination. Analysis of biological samples, including urine, hair and renal tissue, did not provide evidence to support Cd or As toxicity in CKDu patients.

Overall, the observations of this integrated, comprehensive study, which included biological, environmental and pathological investigations, strongly support our previous reports on the absence of Cd and As toxicity in areas with high prevalence of CKDu. Further, these observations do not provide evidence on the involvement of Cd and As in pathogenesis of CKDu in Sri Lanka.

1. Introduction

The prevalence of chronic kidney disease (CKD) in areas in and around North Central Province (NCP) in Sri Lanka is remarkably high when compared with the other parts of the island [1]. The etiology of CKD is not known for the majority of the affected population in this low socio-economic farming community. Thus, this disease entity is known as chronic kidney disease of uncertain etiology (CKDu). The estimated point prevalence of CKDu has been reported to be between 15.1–22.9% in this region following a population based cross-sectional study in 2013 [2] Currently, due to the alarming increase in the number of diagnosed cases, CKDu has been recognized as one of the major non-communicable diseases in Sri Lanka and reducing the CKDu burden is identified as a preventative health goal in the National Health Policy 2016–2025 [3]. Given that CKDu affects predominantly young male farmers in this agricultural community in dry zone regions, the disease

Abbreviations: CKDu, chronic kidney disease of uncertain etiology

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https://doi.org/10.1016/j.jtemb.2019.04.019
Received 18 November 2018; Received in revised form 3 April 2019; Accepted 28 April 2019
0946-672X/

has a huge negative impact on the country's economy [4]. Adding to this, with the available limited facilities to provide the required health care for the affected population in a developing country like Sri Lanka, CKDu has become a burden on the public health system as well.

Over the years, investigations into the etiology of CKDu have reported different hypotheses and identified several risk factors. Since the histopathological evidence suggests CKDu is a tubulointerstitial disease, most investigations were focused on identifying a potential renal toxin. There have been various reports on the possible involvement of various toxins such as heavy metals, mycotoxins, agrochemicals and snake venom [2,5–8]. Among these potential etiological factors, much debate has been on controversial evidence relating to the involvement of Cd and As.

The kidney is an important target organ for environmental toxins. Trace elements such as Cd, Pb and As are environmental toxins which are known to have nephrotoxic effects following acute and chronic exposures [9–11]. Contaminated food and drinking water are the two main sources of these trace elements that enter the human body [10]. Rice is the staple food in Sri Lanka, and given that it can accumulate trace elements such as Cd and As, rice becomes an important dietary source of these elements [10,12]. Thus, drinking water and rice are the two most important environmental exposures that need to be investigated to evaluate possible trace element toxicity in this affected population. In addition to the urinary excretion patterns, accumulation of these elements along with tissue reaction in target organs can provide evidence of possible toxicity [11,13–15].

CKDu was suggested to be an environmentally induced disease in the first epidemiological study carried out in 2007, which aimed to identify potential risk factors of CKDu [16]. The possible involvement of Cd as a causative factor for CKDu came to light with the first report by Bandara et al. in 2008, followed by a second report from the same research group identifying elevated dietary Cd in the affected region [6]. Illeperuma et al. (2009) reported that leaching of Al from inferior quality cooking utensils due to high fluoride levels in water as a culprit [5]. The involvement of Cd on CKDu was further supported by Jayatilake et al. (2013), who reported significantly higher urinary excretion of Cd in individuals with CKDu [2].

In our previous investigations, we could not detect mineral or trace element contamination in drinking water or rice samples collected from the affected region and the observed levels were not significantly different from the levels in unaffected areas in the country [4,17]. The aim of this paper is to summarize the concentrations of trace elements and minerals including Cd and As in environmental and biological samples collected from the affected region to further confirm the absence of trace element induced nephropathy in CKDu. Pathological investigations on CKDu are limited even though pathological characterization provides important evidence on pathogenesis and etiology of the disease. Electron microscopic analysis of renal tissue is also expected to provide further insight into the pathological process and causative factors of CKDu.

2. Materials and methods

Ethical approval for the investigations was obtained by the local human research ethics committees of Kyoto University, Japan, and the Faculty of Medicine, University of Peradeniya, Sri Lanka. All human samples and information were obtained following informed written consent and the study was performed in accordance with the Declaration of Helsinki.

2.1. Sample collection

Twenty-nine drinking water samples from the areas with a high incidence of CKDu were collected into polypropylene bottles that were rinsed with nitric acid and then with purified water before sampling. Water sources were randomly selected to represent different types of water sources used by the CKDu patients and controls in the affected region, such as dug wells, tube wells, surface water sources and treated water. Thirty-eight rice samples from the affected region and sixteen samples from unaffected areas were also collected.

Urine samples (577 samples, from 301 cases and 276 controls) and hair samples (96 samples, from 20 cases and 76 unrelated controls) were collected from both diagnosed CKDu patients and controls from both affected and unaffected areas. CKDu cases were defined as being CKD patients with tubulointerstitial damage that was not a secondary complication of diabetes (history of diabetes mellitus and HbA1c > 6.5% at the time of diagnosis of CKD) or hypertension (history of chronic and/or severe hypertension at the time of diagnosis of CKD). CKD due to other known renal diseases such as autoimmune diseases, glomerular nephritis, Fanconi syndrome or IgA nephropathy (presence of histopathological and immunofluorescence evidence) was also excluded. Volunteer apparently healthy individuals who had no history of diabetes mellitus, hypertension or renal diseases were selected as controls. In the control group, individuals identified with hypertension, diabetes or possible renal impairment through blood pressure measurement (> 140/90 mmHg) and dipstick testing for proteinuria and glycosuria in spot urine samples were excluded from the study. Spot mid-stream urine samples were collected into polypropylene tubes. Hair samples were collected using stainless steel scissors from the nape of the head closer to the scalp. Samples were stored separately in air-tight polyethylene bags. Water, rice and urine samples were immediately stored at 4 °C and then transferred to −30 °C within six hours of collection. The samples were shipped to Japan at −20 °C and stored at −30 °C until analysis.

Renal biopsy specimens were obtained from five randomly selected diagnosed CKDu patients from CKD stage 2 (n = 2) and CKD stage 3 (n = 3) who were having tubulointerstitial disease. Patients from CKD stage 1 were not selected due to the presence of minimum and sometimes nonspecific pathological evidence. Histopathological interpretation at cellular level is difficult in the presence of advanced fibrotic changes of the renal tissue in CKD stages 4 and 5 and obtaining the biopsy samples is difficult due to contracted kidneys. Thus, biopsies from CKD stages 4 and 5 were not included in the present study. The obtained renal specimens were immediately fixed with 2.0% glutaraldehyde, rinsed with 0.1 mol/L cacodylate buffer, fixed in 1% OsO_4, rinsed with 0.1 mol/L cacodylate buffer, and dehydrated in alcohol before embedding.

2.2. Sample analysis

Inductively coupled plasma mass spectrometry (ICP-MS) (Agilent 7700) was used to analyze drinking water, rice and urine samples for trace elements and minerals including Cd and As and hair samples for the concentration of As. All the samples were wet-digested with concentrated nitric acid (Kishida Chemical, Osaka, Japan). Rice and hair samples were pulverized into powders under liquid nitrogen and then added with perchloric acid and nitric acid to be digested. Digested samples were diluted in purified water and analyzed using ICP-MS. A list of respective isotopes used in analysis and instrumental limits of detection (LOD) are provided in Supplementary Table 1. For quality assurance, certified reference materials were included in the analysis and confirmed good agreements with certified levels of concentrations (NMIJ CRM 7402-a No.20; Seronorm Urine L-2). Analyzed values and recovery rates of them are listed in Supplementary Table 2. The fixed renal tissue sections were stained with uranyl acetate and lead citrate for transmission electron microscopy (Hitachi H-7650) observations at the Tazuke Kofukai Medical Research Institute, Kitano Hospital, Osaka, Japan.

2.3. Statistical analysis

Statistical analyses were performed using IBM SPSS Statistics

(version 23, IBM SPSS Inc., Chicago, IL). Based on the normality assessments using the Shapiro-Wilks test, along with visual inspection, parametric and non-parametric statistical tests were used for analyses as required. The distribution pattern of data was presented with means and standard deviations or with the median and interquartile range. Minimum and maximum values have been provided. Based on the normality test results, an independent sample *t*-test (parametric) or the Wilcoxon rank sum test (non-parametric) was used to compare the concentrations of elements between affected and unaffected regions and between cases and controls. The level of significance was set at ≤ 0.05. When calculating the mean values, target analytes with concentrations lower than LOD were considered as one-half of limit of detection. LOD/2 substitution is considered to be adequate, if the detection rate is higher than 85% [18]. Data between LOD and limit of quantification (LOQ) values were used as they were without any substitution.

Principal component analysis was performed as the dimension reduction method using the data of all urine samples (n = 577, 301 cases and 276 controls). Since the ranges of minerals and trace elements detected in the urine samples were not comparable with each other, the unit of the concentration in each element was re-defined for this analysis, to have all the elements in a comparable range with different units. Total variations of urinary elements in the samples were computed using varimax rotated factor analysis by the principal component extraction method. The components with absolute eigenvalue of more than 1 or the 3 components with highest eigenvalues were considered for further analysis. To identify the possible clustering of cases and controls, regression coefficient of each sample was plotted in components 1–2, 1–3 and 2–3.

3. Results and discussion

3.1. Analysis of drinking water

Concentrations of minerals and other elements in drinking water samples collected from both cases and controls in the affected region are listed in Table 1. The concentrations of all 18 tested elements were well within the guidelines set by the World Health Organization (WHO) and Sri Lankan drinking water guidelines [19,20]. Only the concentration of Ca in drinking water collected from cases was closer to meeting the Sri Lankan guidelines for potable water, however, it was still below the recommended value of 100 mg/L [19]. When compared, the mean concentrations of Cr and As were relatively higher in case samples while the mean concentrations of Al, K, Mg, Co, Ni, Zn, Rb, Se, Cu, Mn, Sr, Cd, Ba, Ca, U and Pb were higher in the control samples. These differences were not statistically significant ($p > 0.05$) except for the Cu content in drinking water which was significantly higher in the samples collected from the controls than the samples collected from the cases ($p = 0.02$). It is noteworthy that none of the suspected elements that could be involved in pathogenesis of CKDu, such as As, Cd and Al, demonstrated a statistically significant difference between cases and controls ($p > 0.05$). Further, the maximum concentrations of Cd and Pb in drinking water samples collected from cases that were 0.002 and 0.003 μg/L respectively and were more than 1000 times lower than the recommended upper limits [19,20]. The maximum As concentration reported in drinking water collected from CKDu cases was 1.94 μg/L, which is well below the recommended guideline value of 10 μg/L [19,20].

When compared with the reported values, the mean As level of the samples that were collected from dug wells used by CKDu patients (0.34 μg/L) was considerably lower than the levels reported from previous south Asian groundwater studies. For example, the mean As concentration in groundwater samples collected from Bihar (India) was 62.2 μg/L (range: < 0.01–389.4 μg/L) [21], from Patna (India) it was 52 μg/L [22], and from Uttar Pradesh (India) it was 331 μg/L [23].

3.2. Analysis of rice samples

Table 2 summarizes the observed As, Pb and Cd concentrations in rice with a comparison based on the values reported by previous studies.

3.2.1. As in rice

Comparatively higher bioavailability of As under reduced soil redox potential conditions accelerates the bio accumulation of As in all types of cereals [24]. Rice has been identified as being more efficient at assimilating As into its grain than other staple cereal crops [25]. In this study, the mean total As concentration was 0.053 mg/kg and is comparable with the levels reported for dry zone in Sri Lanka [26]. The As levels in rice reported in this study were not comparable with previous studies that reported As contamination in rice, such as a 0.2 mg/kg level from the USA [27], 0.21 mg/kg from Spain [28] and 0.99 mg/kg from

Table 1
Concentration of minerals and trace elements in drinking water.

Element	Unit	Water used by CKDu cases (n = 16)		Water used by healthy controls (n = 13)		P value	WHO guideline value [20]	Sri Lankan guideline value [19]
		Mean (SD)	Range	Mean (SD)	Range			
Al	μg/L	0.58 (0.48)	0.10-1.5	1.3 (2.2)	< 0.10-7.4	0.26	900	200
K	mg/L	1.2 (1.9)	0.18-8.0	2.4 (5.0)	0.32-21	0.39	*	N/A
Cr	μg/L	0.043 (0.052)	0.010-0.21	0.039 (0.035)	0.011-0.15	0.84	50	50
Mg	mg/L	16 (11)	4.4-48	24 (16)	4.9-57	0.16	N/A	30
Co	μg/L	0.022 (0.008)	0.010-0.039	0.16 (0.55)	0.010-2.2	0.34	N/A	N/A
Ni	μg/L	0.41 (0.31)	0.14-1.0	0.75 (0.97)	0.040-3.4	0.20	70	20
Zn	μg/L	0.58 (0.62)	< 0.010-2.0	0.97 (1.2)	0.040-3.9	0.27	*	3000
As	μg/L	0.34 (0.47)	0.060-1.9	0.18 (0.19)	0.080-0.83	0.22	10	10
Rb	μg/L	0.76 (0.54)	0.22-2.3	2.0 (2.3)	0.16-9.6	0.06	N/A	N/A
Se	μg/L	0.18 (0.30)	0.010-1.1	0.21 (0.16)	0.040-0.58	0.67	40	100
Cu	μg/L	0.26 (0.11)	0.090-0.49	0.47 (0.31)	0.10-1.0	0.02	2000	1000
Mn	μg/L	0.52 (0.57)	0.044-2.4	0.64 (0.65)	0.067-2.1	0.60	*	100
Sr	mg/L	0.15 (0.13)	0.020-0.41	0.32(0.31)	0.042-1.2	0.06	N/A	N/A
Cd	μg/L	0.001 (0.001)	< 0.001-0.002	0.001(0.001)	< 0.001-0.004	0.61	3	3
Ba	mg/L	0.054 (0.051)	0.009-0.17	0.085 (0.055)	0.017-0.18	0.11	1.3	N/A
Ca	mg/L	21 (9.9)	9.0-480	31 (20)	12-90	0.08	N/A	100
U	μg/L	0.058 (0.086)	0.003-0.34	0.060 (0.080)	0.003-0.25	0.95	30	N/A
Pb	μg/L	0.003 (0.001)	< 0.002-0.003	0.012 (0.009)	< 0.002-0.027	0.27	10	10

*Guideline value is not available either because it is not a health concern at levels found in drinking water or occurs in drinking water at concentrations well below those that pose a health concern; N/A, not available. When calculating the mean values, target analytes with concentrations lower than limit of detection (LOD) were considered as one-half of limit of detection [18].

Table 2
A comparison of As, Pb and Cd concentrations in rice samples from CKDu areas in Sri Lanka and other geographical regions.

Element	Geographical region	N	Mean concentration (minimum-maximum) mg/kg	Reference
As	Sri Lanka (CKDu affected)	38	0.053 (< 0.01-0.26)	This study
	Sri Lanka (CKDu unaffected)	81	0.048 (0.002-0.213)	[26]
	China	712	0.119 (< 0.008-0.490)	[30]
	China (Changshu)	155	0.199 (< 0.01-0.587)	[29]
	China (Taizhou)	13	0.155 (0.095-0.308)	[31]
	Taiwan	204	0.080	[32]
	Turkey	25	0.098 (0.0204-0.1708)	[33]
	United States	112	0.20	[27]
	Spain	24	0.21	[28]
Pb	Sri Lanka (CKDu affected)	37	0.026 (0.001-0.15)	This study
	Sri Lanka (CKDu unaffected)	81	0.257 (0.002-1.277)	[26]
	China	712	0.062 (< 0.005-0.400)	[30]
	China (Jiangsu)	23	0.054 (0.0076-0.12)	[34]
	China (Changshu)	155	0.171(< 0.003-0.957)	[29]
	China (Taizhou)	13	2.042 (0.256-2.602)	[31]
	China	269	0.114	[35]
	Iran	67	0.068 (0.040-0.128)	[36]
Cd	Sri Lanka (CKDu affected)	37	0.031 (0.005-0.22)	This study
	Sri Lanka (CKDu unaffected)	81	0.128 (0.0006-0.576)	[26]
	China	712	0.050 (< 0.001-0.740)	[30]
	China (Jiangsu)	23	0.014 (0.005-0.032)	[34]
	China (Changshu)	155	0.019 (< 0.001-0.201)	[29]
	China (Taizhou)	13	0.224 (0.012-0.661)	[31]
	China	269	0.081 (-0.340)	[35]
	Iran	67	0.062 (0.038-0.122)	[36]
	Turkey	25	0.031 (0.0084-0.0775)	[33]

When calculating the mean values, target analytes with concentrations lower than limit of detection (LOD) were considered as one-half of limit of detection [18].

China [29]. The concentration of As in rice directly depends on the condition of the paddy soil, and the results of this study did not provide evidence of As-polluted soil or As contaminated rice in CKDu affected areas.

3.2.2. Cd in rice

The Cd concentration detected in rice samples collected from CKDu affected areas varied from LOD to 0.218 mg/kg with a mean concentration of 0.031 mg/kg. None of the sample levels exceeded the standard level of 0.4 mg/kg, which is recommended by the Codex Committee on Food Additives and Contaminants (CCFAC) [37]. Out of 37 case rice samples analyzed in this study, two samples (5.4% of case samples) had a Cd concentration of more than 0.2 mg/kg (the standard for Cd in rice set by Korea and China) [38] and three samples (8.1% of case samples) exceeded the level of 0.1 mg/kg (the standard for Cd in rice set by the European Union, Australia and New Zealand) [39,40]. The Cd levels reported in this study are comparable with the concentrations reported in other countries, such as China [29,30], Iran [36] and Turkey [33] (Table 2).

3.2.3. Pb in rice

In comparison with the literature data on concentration of Pb in rice in other countries, observed values in this study are relatively low, with a mean concentration of 0.025 mg/kg (0.001 mg/kg to 0.146 mg/kg). In 2008, Fu et al. reported that the mean Pb content in rice samples (n = 13) from Taizhou, China was 2.04 mg/kg [31], which is about 80 times higher than the level reported in this study. Concentration of Pb in rice reported from Iran [36] are also higher than the values observed in this study (Table 2).

Table 3
Estimated weekly intake of Cd, As and Pb via rice and water.

Elements	PTWI μg/kg bw per week	Mean ETWI μg/kg bw per week (this study)	Maximum ETWI μg/kg bw per week (this study)
Cd	5.6	1.1	7.7
Pb	*	0.9	5.2
As	**	2.0	9.7

PTWI; Provisional Tolerable Weekly Intake, ETWI; Estimated Total Weekly Intake, bw; body weight [43,44]; ** Joint FAO/WHO Expert Committee on Food Additives (JECFA) has withdrawn the PTWI guideline value for As and Pb [45–47].

3.3. Estimated intake of Cd, As and Pb

Assuming rice and water are the two main pathways of human exposure to As, Cd and Pb for the target population, the total weekly intake (ETWI) of these elements were estimated (Table 3). The average rice consumption for Sri Lanka (110.2 kg/person/year) was extracted from the special report published by the World Food Program [41] and the maximum and average drinking water consumption were assumed to be 2.5 L/d/person and 2.25 L/d/person respectively [42].

The mean ETWI of Cd was well below the Provisional Tolerable Weekly Intake (PTWI) of 5.6 μg/kg bw recommended by Joint FAO/WHO Expert Committee on Food Additives (JECFA) [43,44]. However, the maximum total weekly intake of Cd estimated for two cases (5.3% of total cases) exceeded the PTWI. Previously established PTWI of 15 μg/kg body weight for As and 25 μg/kg body weight for Pb have been withdrawn by Joint FAO/WHO Expert Committee on Food Additives (JECFA) [45–47]., European Food Safety Authority (EFSA) has provided a benchmark dose level (BMDL) and a corresponding dietary intake value for Pb for effects on CKD ($BMDL_{10}$ = 15 μg/L in blood, corresponding dietary intake = 0.63 μg/kg body weight per day) [48]. Currently, there is no guideline value for tolerable weekly intake for As to compare our observation. However, the observed values are substantially lower than the values reported in As contaminated areas [49,50]

Our calculation of estimated intake levels of Cd, As and Pb via rice and water was based on environmental sample analysis. The present study could not compare the estimated intake levels of these trace elements in CKDu cases with controls.

3.4. Analysis of urine samples

Table 4 summarizes the levels of minerals and trace elements detected in 577 urine samples which was reported in our previous publication [17]. Included cases and controls did not differ statistically based on history of snake bite, history of malaria, smoking and betel chewing habits ($p > 0.05$). However, the family history of CKD was more common among the cases than the controls ($p < 0.05$) [17]. Concentrations of none of the nephrotoxic elements including Cd, As and Pb were higher in urine in cases than in controls ($p < 0.05$).

The top 3 eigenvalues (λ) of each principal component (PC) obtained using the factor reduction method were 377.5, 262.0 and 72.9 respectively. The first component alone accounted for 43.5% of the urinary heavy metal variance among the samples, whereas the second and third components accounted for only 30.2% and 8.4% respectively. Cumulative variance for the third PC was 92%. Top 13 PC resulted in eigenvalues greater than 1. Fig. 1 shows the scatter plots of regression factor score 1–2, 1–3 and 2–3. Lower concentrations of elements in case samples than control samples were reflected in reduced dimension analysis by demarcating a cluster boundary to encage > 80% of the case samples. This observation suggests a potential application of combined urinary mineral and trace element levels as a CKDu marker which needs further investigation. The same regression factor scores were replotted incorporating the details of drinking water sources.

Table 4
Concentration of minerals and trace elements in urine [17].

Metal	Cases (μg/l) n = 301			Controls (μg/l) n = 276			p^avalue	p^bvalue
	Mean (SD)	Min − Max	25% / 50% / 75%	Mean (SD)	Min − Max	25% / 50% / 75%		
Pb	0.94 (0.89)	0.05–8.42	0.39 / 0.68 / 1.16	1.88 (2.55)	0.21–31.8	0.96 / 1.43 / 2.23	< 0.001*	< 0.001*
Tl	0.18 (0.19)	0–1.10	0.06 / 0.11 / 0.22	0.38 (0.26)	0.01–1.73	0.2 / 0.32 / 0.49	< 0.001*	< 0.001*
Cs	3.09 (2.77)	0.25–20.3	1.13 / 2.16 / 4.23	5.81 (4.08)	0.14–23.6	2.92 / 4.87 / 6.99	< 0.001*	< 0.001*
Cd	0.47 (0.78)	0.02–7.17	0.11 / 0.25 / 0.52	1.76 (1.89)	0.03–8.24	0.23 / 0.78 / 3.1	< 0.001*	< 0.001*
Mo	55.6 (48.9)	2.36–314	19.4 / 42.6 / 75.9	105 (97.5)	4.62–972	39.3 / 80.8 / 141	< 0.001*	< 0.001*
Sr	132 (115)	8.74–650	55.4 / 97.3 / 172	247 (163)	16.1–829	119 / 226 / 332	< 0.001*	< 0.001*
Rb	1,380 (1,250)	185–9,860	575 / 1,030 / 1,780	2,360 (1620)	130–9,900	1,270 / 1,980 / 2,810	< 0.001*	< 0.001*
Se	16.8 (15.0)	0.37–101	6.11 / 12.8 / 22.5	29.8 (20.0)	2.00–124	15.9 / 25.4 / 38.2	< 0.001*	< 0.001*
As	33.2 (37.5)	1.49–259	10.2 / 21.8 / 40.6	44.4 (43.5)	1.22–277	18.1 / 30.2 / 55.4	< 0.001*	< 0.001*
Zn	374 (322)	36.2–2,130	163 / 276 / 476	428 (453)	24.6–4,210	180 / 315 / 522	0.1	> 0.10
Cu	16.2 (15.5)	0.99–153	7.70 / 11.9 / 19.3	15.7 (11.6)	1.41–116	8.39 / 13.1 / 19.9	0.628	> 0.10
Ni	4.04 (3.06)	0.18–23.2	1.79 / 3.18 / 5.27	5.52 (4.19)	0.45–37.9	3.07 / 4.54 / 6.92	< 0.001*	< 0.001*
Co	0.42 (0.41)	0.04–3.30	0.17 / 0.32 / 0.48	0.55 (0.48)	0.03–3.28	0.26 / 0.42 / 0.65	< 0.001*	< 0.001*
Fe	10.8 (24.4)	0.04–280	3.37 / 5.89 / 10.4	11.5 (46.9)	1.36–773.18	4.3 / 6.59 / 9.86	0.817	< 0.025
Mn	0.68 (1.14)	0.07–19.0	0.34 / 0.53 / 0.75	0.84 (1.97)	0.08–31.9	0.38 / 0.60 / 0.90	0.213	< 0.05
Cr	0.19 (0.30)	0–3.62	0.08 / 0.13 / 0.19	0.24 (0.78)	0.01–12.63	0.10 / 0.15 / 0.23	0.291	< 0.005
V	0.46 (0.31)	0.02–1.66	0.24 / 0.41 / 0.61	0.67 (0.31)	0.01–2.67	0.46 / 0.68 / 0.86	< 0.001*	< 0.001*
Al	4.60 (3.61)	0.31–30.0	2.54 / 3.67 / 5.52	6.60 (8.51)	0.03–114.98	3.37 / 4.78 / 7.39	< 0.001*	< 0.001*

p^a value calculated by *t*-test; p^b value calculated by Kolmogorov-Smirnov (non-parametric) test; *Significant by Bonferroni correction ($p = 0.05/18 = 0.003$).

However, clusters were not identified for different sources of drinking water, suggesting a minimum impact of the source of drinking water on the levels of urinary elements.

3.5. Analysis of hair samples

Based on published data, As plays an important role in several reported hypotheses on pathogenesis of CKDu in Sri Lanka. Hair As concentration is considered as a reliable indicator of chronic As exposure [51].

Table 5 summarizes As concentrations in hair samples collected from CKDu cases in affected regions and controls from both affected and unaffected regions in the country.

The mean concentration observed in cases was lower than the concentrations in both control groups and all observed values were lower than the level reported in the non-exposed adult population (< 1 μg/g) [52]. The maximum concentration of As in 93 hair samples analyzed in this study was 0.94 μg/g while it was only 0.17 μg/g for CKDu cases. The observed As concentrations were comparable with the values reported in populations with no exposure to As [53–56]. Further, the hair As concentrations reported in this study were well below the levels reported from As contaminated areas such as West Bengal [57], Vietnam [58] and Cambodia [59]. The observed values did not provide any evidence on As toxicity in affected or the unaffected areas from where the hair samples were collected.

3.6. Electron microscopic analysis of renal tissue

The clinical and demographic characteristics of five CKDu patients selected are shown in Table 6. The mean age of the patients was 43.0 ± 6.3 years (range 34–51 years). The mean systolic blood pressure was 124.6 ± 14.9 mmHg and the mean diastolic blood pressure was 72.6 ± 7.6 mmHg. Two out of the five patients were overweight (BMI > 25 kg/m^2) while one patient was underweight (BMI < 18.5 kg/m^2). Out of the five patients, two had a family history of CKD. In overall observations, both kidneys appeared to be slightly contracted when compared with reported values for normal healthy adults. Cortico-medullary demarcations were present on ultrasonographic examination of the kidneys of all patients except in two patients in CKD stage 3.

CKD, chronic kidney disease; BMI, body mass index; eGFR, estimated glomerular filtration rate; USS, ultrasound scan.

Electron microscopic analysis of relatively early stages of CKDu revealed mild wrinkling of the basement membrane and subendothelial edema with effacement of foot processes in the segmental region of the glomerulus (Fig. 2). None of the samples had electron-dense deposits in the glomeruli or tubulointerstitium. Partial tubular cell degeneration was observed in the renal tissue. Neither dysmorphic mitochondria nor accumulation of metallothioneins which can be potential indicators of Cd and As toxicity were found in the cytoplasm of the proximal tubular epithelial cells in any of the samples (Fig. 3).

In long term exposure, metals like Cd and As are known to cause renal tubular dysfunction through oxidative stress [13,60,61]. In addition, these elements induce over-expression of metallothioneins in

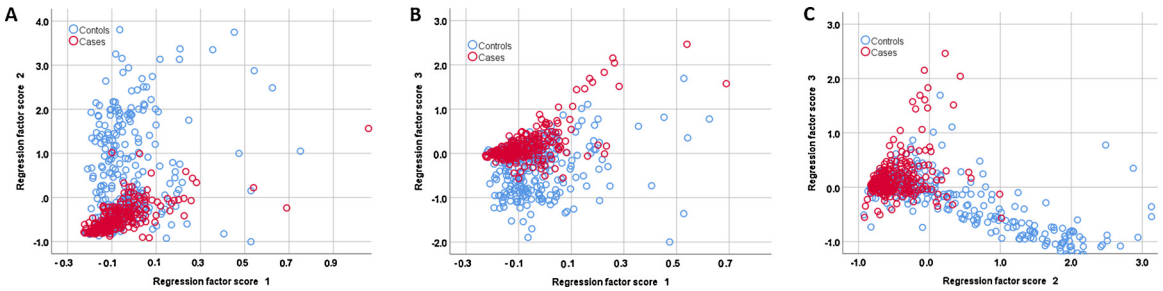

Fig. 1. Scatter plots of regression factor scores.
Regression factor score 1–2 (A), 1–3 (B) and 2–3 (C) estimated with factor reduction method. Red circles denote the cases and blue circles denote the controls. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

Table 5
As concentration in hair samples.

	n	Mean (SD) (μg/g)	Minimum-Maximum (μg/g)	95% Confidence Interval
CKDu Cases	20	0.05 (0.04)	0.001-0.17	0.03-0.07
Controls (affected region)	55	0.12 (0.15)	0.001-0.94	0.08-0.16
Controls (unaffected region)	18	0.23 (0.20)	0.001-0.94	0.13-0.33

Table 6
Clinical and demographic features of the patients who participated in the electron microscopy study.

Ref ID	BMI (kg/m^2)	eGFR (mL/min/1.73 m^2)	Blood pressure (mmHg)	Age (yrs)	USS of the kidneys		Cortico-medullary demarcation	
					Right	Left	Right	Left
1	21.30	36.57	101/62	51	8.9*4.0	8.8*4.6	lost	poor
2	25.83	46.99	132/68	43	8.9*3.3	8.4*4.1	lost	lost
3	16.75	61.66	130/74	34	8.3*3.7	8.7*3.8	present	present
4	25.50	54.82	142/79	41	9.1*4.6	9.4*4.4	present	present
5	22.31	69.20	120/80	46	8.5*3.8	8.2*4.9	present	present

tissues as a protective mechanism because metallothioneins is a protein that acts as an intracellular scavenger of metals like Cd and protects the kidney from the toxic effects of these metals [13,15]. Therefore, in addition to the metal deposits, over-expression of metallothioneins can be an indicator of Cd and As toxicity [13–15]. Observations of this study indicating the absence of metal deposits or over expression of metallothioneins in renal tissues of CKDu patients indicate that it is unlikely for Cd and As toxicity to be the cause of CKDu.

It is important to report that even though the absence of a history of uncontrolled hypertension at the time of diagnosis of CKDu was one of the criteria to diagnose CKDu patients, some of the glomerular pathological changes observed through this analysis mimicked the changes in renal tissue in the early stages of hypertensive nephrosclerosis [62]. Unfortunately, none of the biopsy samples available for this study were suitable to analyze the pathological characteristics of the renal vasculature to comment on vascular pathology.

4. Conclusion

Despite investigations over several years, the etiology of CKDu in Sri Lanka remains a mystery. Controversial evidence on Cd and As toxicity warranted further investigation to confirm their involvement in pathogenesis of CKDu. The present comprehensive study utilized pathological, biological and environmental samples to systematically assess the exposure to minerals and trace elements including Cd and As in the population living in CKDu affected areas. Mineral and trace element content in drinking water and rice samples collected from the affected region were comparable to the levels observed in non-affected regions and were within the recommended guideline values. Biological samples including urine, hair and renal tissue analyses did not provide any

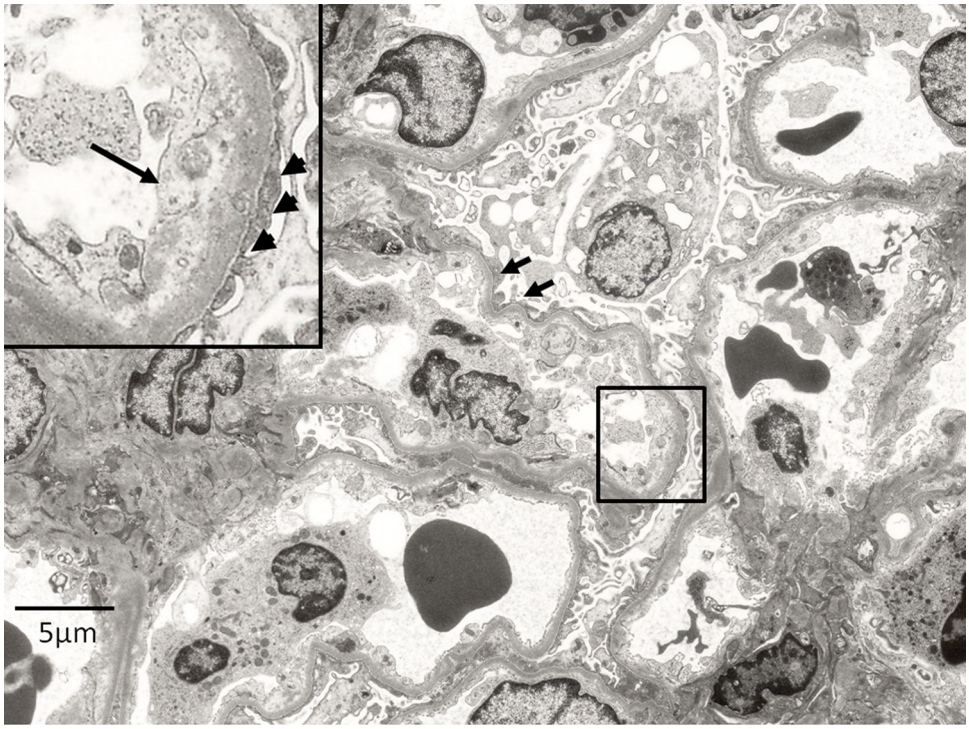

Fig. 2. Electron microscopic picture of a part of a glomerulus.
Mild wrinkling of the basement membrane (arrow head), subendothelial edema (long arrow) and partial effacement of foot processes (short arrow) can be seen. Magnification: ×3000 overall; ×12,000 in the inset.

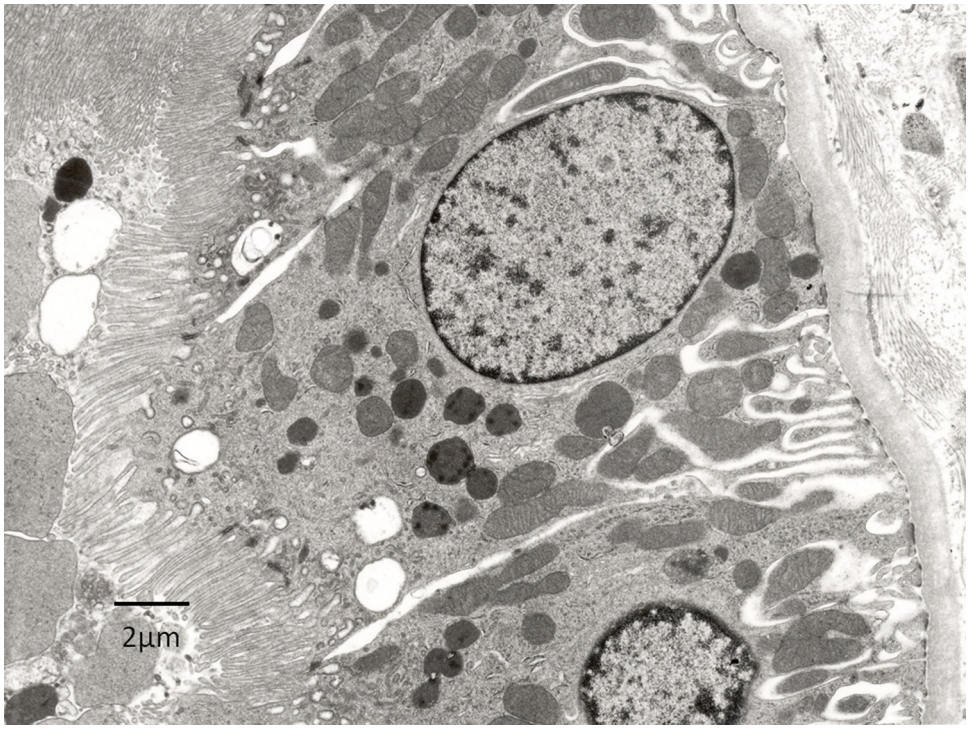

Fig. 3. Cytoplasm of a proximal tubular epithelial cell.
Intact brush boarder and mitochondria and absence of metal deposits can be seen.

evidence on toxicity of trace elements including Cd and As in the CKDu population and their direct contribution to the pathogenesis of CKDu. Overall, similar to our previous observations, this comprehensive analysis did not provide any supportive evidence on trace element toxicity in the CKDu population and did not support a Cd or As induced nephropathy. Thus, the observations of this study can rule out the hypotheses based on direct toxicity of these investigated elements as etiological factors of CKDu in Sri Lanka.

Competing interests

The authors declare that they have no conflicts of interest.

Acknowledgements

This work was supported by the Japan Society for Promotion of Science (23.2361 and 25305021) and Special Coordination Funds for Promoting Science and Technology from the Ministry of Education, Culture, Sports, Science and Technology in Japan (20101410). The funding agencies had no role in the study design, data collection and analysis, decision to publish, or preparation of the manuscript. Authors would also like to acknowledge Prof Hiroshi Yamauchi of Kitasato University, Tokyo, Japan, Dr Toshiyuki Komiya of Kansai Electric Power Hospital, Osaka, Japan and Prof N Ratnatunga of University of Peradeniya, Sri Lanka for their contribution in sample collection and analysis.

Appendix A. Supplementary data

Supplementary material related to this article can be found, in the online version, at doi:https://doi.org/10.1016/j.jtemb.2019.04.019.

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