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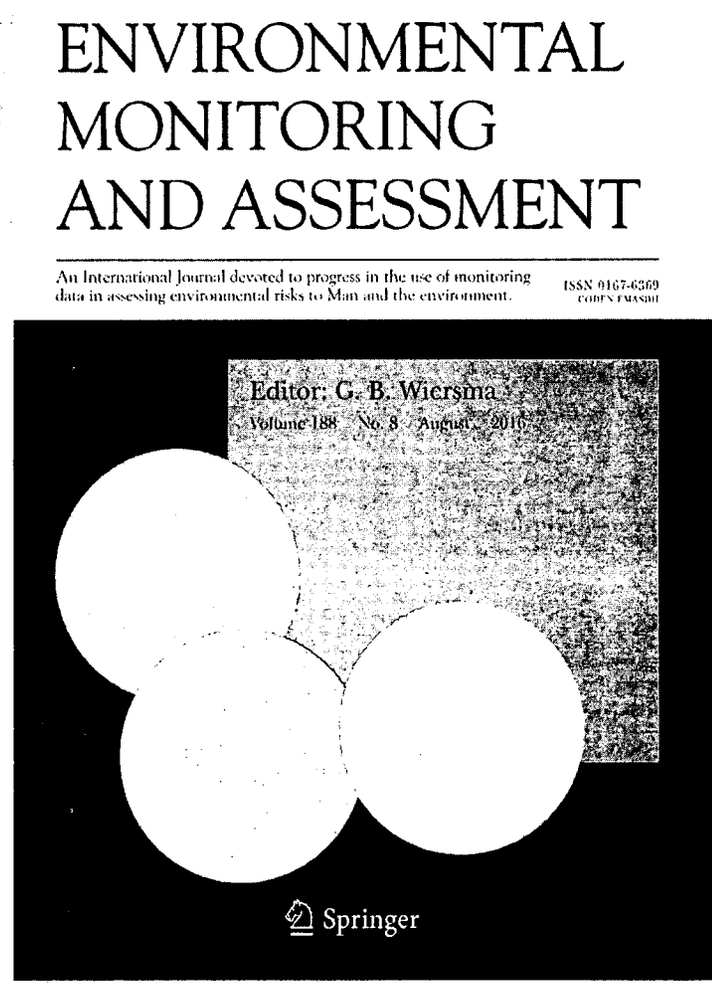

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Quest to identify geochemical risk factors associated with chronic kidney disease of unknown etiology (CKDu) in an endemic region of Sri Lanka—a multimedia laboratory analysis of biological, food, and environmental samples

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Abstract The emergence of a new form of chronic kidney disease of unknown etiology (CKDu) in Sri Lanka's North Central Province (NCP) has become a catastrophic health crisis. CKDu is characterized as slowly progressing, irreversible, and asymptomatic until late stages and, importantly, not attributed to diabetes, hypertension, or other known risk factors. It is postulated that the etiology of CKDu is multifactorial, involving genetic predisposition, nutritional and dehydration status, exposure to one or more environmental nephrotoxins, and lifestyle factors. The objective of this limited geochemical laboratory analysis was to determine the concentration of a suite of heavy metals and trace element nutrients in biological samples (human whole blood and hair) and environmental samples (drinking water, rice, soil, and freshwater fish) collected from two towns within the endemic NCP region in 2012 and 2013. This broad panel, metallomics/mineralomics approach was used to shed light on potential geochemical risk factors associated with CKDu. Based on prior literature documentation of potential nephrotoxins that may play a role in the genesis and progression of CKDu,

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heavy metals and fluoride were selected for analysis. The geochemical concentrations in biological and environmental media areas were quantified. Basic statistical measurements were subsequently used to compare media against applicable benchmark values, such as US soil screening levels. Cadmium, lead, and mercury were detected at concentrations exceeding US reference values in many of the biological samples, suggesting that study participants are subjected to chronic, low-level exposure to these elements. Within the limited number of environmental media samples, arsenic was determined to exceed initial risk screening and background concentration values in soil, while data collected from drinking water samples reflected the unique hydrogeochemistry of the region, including the prevalence of hard or very hard water, and fluoride, iron, manganese, sodium, and lead exceeding applicable drinking water standards in some instances. Current literature suggests that the etiology of CKDu is likely multifactorial, with no single biological or hydrogeochemical parameter directly related to disease genesis and progression. This preliminary screening identified that specific constituents may be present above levels of concern, but does not compare results against specific kidney toxicity values or cumulative risk related to a multifactorial disease process. The data collected from this limited investigation are intended to be used in the subsequent study design of a comprehensive and multifactorial etiological study of CKDu risk factors that includes sample collection, individual surveys, and laboratory analyses to more fully evaluate the potential environmental, behavioral, genetic, and lifestyle risk factors associated with CKDu.

Keywords CKDu · Sri Lanka · Non-communicable disease · Chronic kidney disease · CKD of unknown etiology · North Central Province · Risk factors · Biological media · Environmental media · CKD of non-traditional causes · CKDnT · Geochemical · Regional laboratory analysis · Cadmium · Arsenic · Lead · Mercury · Fluoride · Metals

Introduction

Chronic kidney disease (CKD) has emerged as an important non-communicable, global public health epidemic. Importantly, CKD has become more prevalent in the developing world where the diagnosis and treatment of renal disease is consuming a significant and growing proportion of available healthcare resources (Remuzzi and Horton 2013; Gorry 2013; Garcia-Garcia et al. 2013). Of particular focus for this paper, the emergence of a new form of CKD of unknown etiology (CKDu) that is not attributed to hypertension, diabetes, obesity, or other risk factors typically associated with traditional CKD has further challenged developing nations struggling to address this issue (Cusumano and Bedat 2008). In addition, many patients impacted by emerging CKDu in the developing world are in the prime of their adult working years (30–60 years old), significantly compounding societal impact (Elledge et al. 2014).

Geographical hot spots of CKDu have emerged in a number of countries and several different names have arisen for the epidemic. In Latin America, rural inhabitants in localized areas within El Salvador (Peraza et al. 2012; Garcia-Trabanino et al. 2005; Orantes et al. 2011), Nicaragua (Torres et al. 2010; O'Donnell et al. 2011; Laux et al. 2012), and Mexico (Gutierrez-Amavizca et al. 2013), have been experiencing a growing epidemic for a number of years. Distinguishing characteristics of CKDu in this region, which is also known in this region as CKD of non-traditional causes, or CKDnT, include prevalence among young, male agricultural workers with a history of manual labor in sugarcane or cotton farming under very hot conditions and limited drinking water intake, low-grade proteinuria, an asymptomatic but progressive decline in glomerular filtration rate (GFR), and biochemical markers and biopsy data consistent with renal tubulointerstitial disease. Recent research in this region has focused on how dehydration may play a role in the disease (Roncal-Jimenez et al. 2015). These commonalties also prompted naming of the disease (Mesoamerican nephropathy, MeN) at a recent workshop (Correa-Rotter et al. 2014; Brooks et al. 2012; Wijkstrom et al. 2013).

India is another important hot spot (Agarwal 2005; Siddarth et al. 2013; Siddarth et al. 2014), where the relative scope and pattern of cases with unknown etiology in the Srikakulam district of Andra Pradesh and elsewhere is similar to that observed in agricultural communities in Sri Lanka and Central America (Rajapurkar et al. 2012; Mittal et al. 1997). Balkan endemic nephropathy (BEN) (Long et al. 2001; Jankovic et al. 2011) has been impacting thousands of people in Serbia and other localized areas of the Balkan Peninsula for over 50 years, and chronic interstitial

nephropathy (CIN) (Abid et al. 2003) has been described in Tunisia.

In the 1990s, CKDu began to appear in the North Central Province (NCP) of Sri Lanka (Jayatilake et al. 2013). As with other emerging CKDu epidemics, it is slowly progressive, irreversible, and largely asymptomatic until its later stages (Redmon et al. 2014). It has primarily impacted people of lower socioeconomic status, notably those involved in farming or living in agricultural areas (K. P. Wanigasuriya et al. 2011). A consensus from histopathological and biochemical measurements has emerged that CKDu is characterized by renal tubular and interstitial damage (Nanayakkara et al. 2012a; Nanayakkara et al. 2012b; Wijetunge et al. 2013; Athuraliya et al. 2011). The tubulointerstitial progression of the disease and its restriction to specific areas within the NCP endemic region suggest that the CKDu may result from the cumulative risk caused by multiple potential risk factors, including dehydrating working conditions, exposure to one or more environmental nephrotoxins, genetic susceptibility, and lifestyle factors that could further stress the kidneys (Nanayakkara et al. 2014).

In an effort to determine both the extent and potential risk factors of CKDu in Sri Lanka's NCP, a number of etiological studies have recently been conducted (Wickremasinghe et al. 2011). Laboratory screening studies have been completed to determine the presence of nephrotoxic mycotoxins, including ochratoxin A, in food and biological samples obtained from CKDu patients in the region (Wanigasuriya et al. 2008; Desalegn et al. 2011), and investigators have also explored the association between potential exposure to acetylcholinesterase (AChE) inhibiting organophosphate pesticides with CKDu (Peiris-John et al. 2006). Another group hypothesized a link between metal-chelating glyophosate and CKDu genesis (Jayasumana et al. 2014). The deficiency of glucose-6-phosphate dehydrogenase (G6PD), an enzyme that protects against oxidative stress, was the focus of another CKDu investigation (Jayasekara et al. 2013).

Additionally, exploratory studies have focused on cadmium exposure in Sri Lanka, as it is a known nephrotoxic environmental contaminant (Sabath and Robles-Osorio 2012) with cited reports of renal tubular dysfunction following cadmium exposure from contaminated rice, water, or other sources in China (Nordberg et al. 2002) and Japan (Kobayashi et al. 2009b, 2009a; Watanabe et al. 2002). Rice and freshwater fish are crucial dietary components for the rural people living within the NCP (Allinson et al. 2002; Allinson et al. 2009; Premarathna et al. 2011), prompting several studies aimed at determining cadmium levels in these matrices (Bandara et al. 2007; Bandara et al. 2010a) and in local area irrigation and drinking waters (Bandara et al. 2010b; Bandara et al. 2011).

Arsenic has also received attention as a potential constituent of concern in the scientific literature (Jayasumana et al. 2013) and popular press (Jayawardana 12 May, 2013). The use of herbal medicines has been associated with chronic kidney disease elsewhere (Soderland et al. 2010; Jha 2010), and a local study identified Ayurvedic medicine use as a CKDu risk factor (Wanigasuriya et al. 2007). Nevertheless, the Sri Lankan research studies completed to date have been limited by analytical method capabilities, small sample sizes, or incomplete biological or environmental media.

Interestingly, the NCP is in Sri Lanka's dry zone and is characterized by complex hydrogeochemistry. Alternating wet and dry cycles can impact agricultural soil redox chemistry and other physiochemical characteristics that determine heavy metal mobility and bioavailability (Wang et al. 2011; Andreu and Gimeno-Garcia 1999). The endemic CKDu area also overlaps with a region of moderate to highly elevated ground fluoride content (Wanigasuriya 2012). Some investigators have speculated that the presence of this elevated fluoride content in combination with other analytes, such as sodium and calcium (Chandrajith et al. 2011) or aluminum in substandard cookware (Illeperuma et al. 2009), can contribute to renal damage. In addition, nutritional status (Senevirathna et al. 2012) and genetic susceptibility (Nanayakkara et al. 2014) of individuals living within the NCP can play a major role in the genesis and ultimate progression of CKDu.

In sum, various studies that focused on a variety of potential risk factors for CKDu have been conducted in Sri Lanka and other CKDu hot spots, but a single hydrogeochemical condition, environmental nephrotoxin, biochemical parameter, or other risk factor has not yet been identified and clearly linked to CKDu. It is postulated that the etiology of CKDu is multifactorial, involving genetic predisposition, nutritional and hydration/dehydration status, and exposure to one or more environmental nephrotoxins. The objective of this limited geochemical laboratory analysis was to determine the concentration of a suite of heavy metals and trace element nutrients in biological (human whole

blood and hair), food (rice and freshwater fish), and environmental (drinking water and soil) samples collected from two towns within the endemic NCP region. This broad panel, metallomics/mineralomics approach was used to shed light on potential geochemical risk factors associated with CKDu. Heavy metals and fluoride were selected for analysis as many literature reports have suggested one or more of these trace elements may play a role in the genesis and progression of the disease. The geochemical concentrations in biological and environmental media from the endemic areas were quantified. Basic statistical measurements were subsequently used to compare media against applicable benchmark values, such as US soil screening levels. This preliminary screening allows us to determine whether specific constituents may be present above general levels of concern, which could be later used in the formation of a more comprehensive study specifically linking nephrotoxins to CKDu.

Field research design and sample collection were completed in 2012 and 2013 through a partnership between the Faculty of Medicine, University of Kelaniya; the Faculty of Medical Sciences, University of Sri Jayewardenepura; the University of Alabama at Birmingham's Department of Epidemiology; and the Atomic Energy Authority, Sri Lanka. After sample collection was completed, RTI International was invited to join the collaboration in late 2013 to support advanced laboratory analysis, data analysis, and additional literature review. This paper presents the results of the multimedia biological and environmental laboratory analyses.

Materials and methods

Study design

The Sri Lanka research team designed and completed multimedia biological and environmental samples collection for this regional geochemical analysis from the NCP of Sri Lanka in 2012 and 2013. Two towns, Medawachchiya and Medirigiriya, were selected within this endemic area for sample collection (Fig. 1). Biological samples (whole blood and hair) were collected from men ranging in age from 29 to 62 years old. Environmental samples were also collected, including from freshwater fish, rice from local mills, drinking water from residences of study participants, and soil samples from the environs of study participant households from within the study area. The study participant recruitment process, informed consent, and study protocol were approved by the Ethics Review Committee of the Faculty of Medicine, University of Kelaniya. RTI International (USA) joined the collaboration, and completed advanced laboratory analyses between November 2013 and March 2014 in blinded fashion with approval from the RTI Institutional Review Board.

Sample collection

Blood (n = 83) and hair (n = 56) samples were collected from patient participants within the study area. Upon collection, blood samples were stored frozen (nominal −20 °C) until analysis, while hair samples were cleaned with a surfactant solution to remove exogenous contamination before storage at ambient temperature. Locally grown rice samples (n = 41) were obtained from area mills and stored at ambient temperature after milling. Commonly consumed freshwater fish (n = 11) collected from study area reservoirs were filleted, and muscle tissue homogenized, lyophilized, and stored at ambient temperature. Composite surface soil samples (n = 18) were collected from 6 to 12 in. below ground surface at a distance of 10 m from the front door of participant households for consistency and to gain an understanding of potential soil contaminants that may be tracked within a house. Collected soil (approximately 500 g) was dried, screened with 2 mm mesh, and stored at ambient temperature. Sediment-free drinking water samples (approximately 1 L, n = 97) were collected using a bucket lowered to a depth of 1 m below water surface in the center of the well from study participant households. Water samples were then stored frozen (nominal −20 °C) until analysis after stabilization with nitric acid (HNO_3).

Sample collection, storage (including blood tubes), and transfer labware was screened to assess potential contamination by extracting overnight at ambient temperature in 2 % (v/v) nitric acid (HNO_3 - J.T. Baker, Center Valley, PA, USA) in high-purity (~18 MΩ) deionized water (DI H_2O, Pure Water Solutions, Hillsborough, NC, USA). Extracts were analyzed against a 10-ng/mL multi-element standard (High Purity Standards, Charleston, SC, USA) traceable to the National Institute of Standards and Technology (NIST) by inductively coupled plasma mass spectrometry (ICP-MS, Thermo X-Series 2, Waltham, MA, USA). Labware was found to be free of significant contamination.

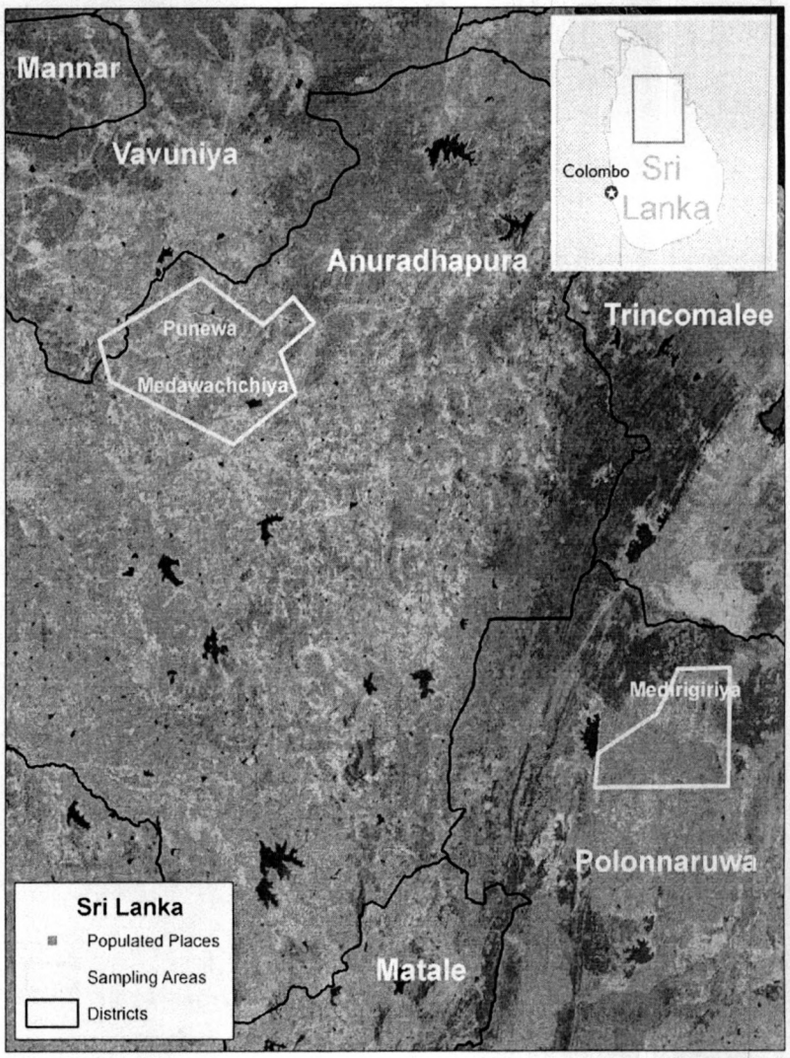

Fig. 1 Selected sampling areas within the CKDu endemic region in Sri Lanka

Heavy metal and trace element analysis

For all study matrices, a broad panel, metallomics/mineralomics approach was utilized. High-purity (Ultrex Grade, J.T. Baker) acids, oxidants (30 % (*v*/*v*), non-stabilized hydrogen peroxide, H_2O_2), and reagents were used, and samples were prepared in a high-efficiency particulate air (HEPA)-filtered environment to minimize contamination. Labware was acid-cleaned and screened to ensure suitability prior to use. ICP-MS and inductively coupled plasma optical emission spectrometry (ICP-OES, Thermo iCAP 6500, Waltham MA, USA) were used to determine concentrations of arsenic (As), cadmium (Cd), copper (Cu), chromium (Cr), lead (Pb), mercury (Hg), nickel (Ni), selenium (Se), tin (Sn), calcium (Ca), iron (Fe), magnesium (Mg), manganese (Mn), and zinc (Zn). Combustion atomic absorption spectrometry (CAAS, Milestone DMA-80, Shelton, CT, USA) was used to determine mercury in rice, fish, and soil matrices, while ion chromatography (IC, ICS3000, Dionex, Sunnyvale, CA, USA) was used to measure fluoride (F) in water samples. Instrumental parameters were selected to maximize sensitivity and minimize interferences (i.e., ICP-MS collision cell gases).

Rigorous quality control (QC) procedures were used for all laboratory measurements. NIST-traceable solutions were used to prepare standards, calibration curves contained at least six acid matrix-matched calibration standards and one blank, and determined concentrations of non-blank standards were required to fall within ±15 % of nominal values. If measured values exceeded the calibration range for an analyte, they were diluted and reanalyzed. To monitor instrument stability, a mid-level standard was analyzed after calibration, at the end of analysis, and after every ten samples. Determined

Table 1 Elemental content in whole blood samples from CKDu endemic area

Analyte	Determined concentration (μg/L)		
	Min.	Max.	Mean
As	<LOD[a]	<LOD	<LOD
Ca	49,300	78,000	64,100
Cd	<LOD	3.11	0.949
Cr	<LOD	218	18.5
Cu	656	1280	942
Fe	302,000	576,000	432,000
Mg	21,400	52,900	33,100
Mn	<LOD	<LOD	<LOD
Ni	<LOD	107	7.28
Pb	1.03[b]	9.09[b]	3.60[b]
Se	172	834	388
Sn	1.8	1.8	1.8
Zn	3020	7150	5580

[a] Determined concentration less than limit of detection (LOD): As = 20.0 μg/L, Cd = 0.250 μg/L, Cr = 3.50 μg/L, Mn = 50.0 μg/L, Ni = 3.00 μg/L

[b] Pb data are reported as micrograms per deciliter

Table 2 Elemental content in hair samples from CKDu endemic area

Analyte	Determined concentration (μg/g)		
	Min.	Max.	Mean
As	<LOD[a]	0.355	NA
Ca	169	3.23	917
Cd	0.0016	0.148	0.0205
Cr	0.0289	3.16	0.245
Fe	6.18	16.8	42.3
Hg	0.0479	212	0.978
K	2.23	5.48	139
Mg	21.6	575	126
Mn	0.278	26.1	6.41
Na	9.93	1640	347
Ni	0.0397	2.65	0.336
Pb	0.0793	14.8	2.07
Sn	0.0179	1.24	0.179
Zn	40.0	270	111

NA not applicable; mean not calculated

[a] Determined concentration less than limit of detection (LOD), 0.0186 μg/g; LODs varied by sample mass; $n = 51$ samples were < LOD

analyte concentrations were required to be within ±20 % of nominal values for bracketed data to be valid. Multiple reagent blanks were processed with each batch to assess analyte contribution from the reagents and the procedure, and standard or certified reference materials (SRMs or CRMs) were used to monitor accuracy and precision. A brief description of the laboratory methods employed for each medium is provided below.

Blood sample preparation and analysis

Blood was removed from freezer storage, allowed to reach room temperature, and gently vortexed prior to removal of nominal 0.5000-g aliquots (~0.5 mL). Aliquots were transferred to 50-mL acid-cleaned tubes, and HNO_3 (2.50 mL), HCl (0.125 mL), and H_2O_2 (0.50 mL) were added. The tubes were ramped to 110 °C in a digitally controlled hot block (SCP Science, Champlain, NY, USA) over 3 h and held at this temperature for 1 h. After cooling, the samples were brought to 25 mL with DI H_2O for analysis. As, Cd, Cu, Cr, Pb, Ni, Se, and Sn were determined by ICP-MS; Ca, Fe, Mg, Mn, and Zn were measured by ICP-OES. Duplicate aliquots of NIST 955c Caprine Blood were prepared and analyzed with the study samples.

Hair sample preparation and analysis

Surfactant-cleaned hair samples were transferred to glass vials and treated with 0.5 mL of acetone (Burdick and Jackson, HPLC Grade, Morris Plains, NJ, USA) to ensure complete removal of exogenous contamination. Acetone was decanted and hair was allowed to dry under HEPA-filtered air. All available samples were analyzed (0.0006 to 0.1607 g; median of 0.0641 g). HNO_3 (0.500 mL), hydrochloric acid (HCl; 0.200 mL), DI H_2O (0.500 mL), and 1000 μg/mL gold (Au; 0.025 mL) were added, then samples were ramped to 90 °C and held at this temperature for 2 h. The vials were allowed to cool to room temperature before adding H_2O_2 (0.500 mL), ramping to 110 °C and holding for 30 min. The samples were brought to 5 mL volume with DI H_2O. Aliquots ($n = 5$) of a human hair certified reference material (GBW 09101, People's Republic of China) were prepared and analyzed with the study samples. As, Ca, Cd, Cu, Cr, Fe, Hg, K, Mg, Mn, Na, Ni, Pb, Sn, and Zn were determined by ICP-MS.

Table 3 Element content in rice samples from CKDu endemic area

Analyte	Determined concentration (mg/kg)		
	Min.	Max.	Mean
Cd	0.00520	0.117	0.0412
Cr	0.00615	0.179	0.0238
Cu	0.833	5.43	3.11
Fe	1.25	70.8	11.4
Hg	0.00220	0.381	0.0368
K	409	2150	1320
Mg	61.1	1120	387
Mn	3.00	37.2	11.3
Na	14.8	86.3	41.9
Ni	0.0725	1.34	0.330
Pb	0.00796	0.146	0.0286
Sn	0.000722	0.0324	0.00668
Zn	10.1	31.1	22.3

Rice sample preparation and analysis

For ICP measurements, the samples were mixed with a plastic spatula to ensure homogeneity prior to aliquot (nominal 0.5000 g) removal. The aliquots were transferred to 10-mL glass tubes, and HNO_3 (1.00 mL), HCl (0.250 mL), and H_2O_2 (0.250 mL) were added, with a Teflon-coated stir bar. Samples were placed in a SPD Discover microwave digestion system (CEM Corporation, Matthews, NC, USA), ramped to 175 °C and held at temperature for 5 min. After cooling to room temperature, the samples were brought to 10 mL volume with DI H_2O and centrifuged at 3500 rpm (Beckman GS-6, Brea, CA, USA) to pelletize remaining material. Triplicate aliquots of NIST 1568a (Rice Flour) were processed with the study samples. For CAAS analysis, nominal 0.0400-g aliquots were transferred to combustion cuvettes and ramped to 800 °C. NIST SRM 2709a San Joaquin Soil and TORT-2 Lobster Hepatopancreas CRM (n = 9, National Research Council of Canada, NRCC) were used to calibrate the CAAS and monitor method performance, respectively. Cd, Cu, Cr, Pb, Mn, Ni, Sn, and Zn were determined by ICP-MS, Fe, Mg, K, and Na by ICP-OES, and Hg by CAAS.

Fish sample preparation and analysis

For ICP analyses, samples were mixed with a plastic spatula prior to aliquot (nominal 0.1000 g) removal. Aliquots were transferred to 10-mL glass tubes, HNO_3 (1.50 mL) was added, and the samples were placed in a digitally controlled hot block maintained at 75 °C for 1 h. HCl (0.50 mL) was added, and temperature was gradually ramped to 180 °C and held for 10 min. After cooling to room temperature, the samples were brought to 10 mL with DI H_2O. Triplicate DORM-1 (Dogfish Muscle) and DOLT-3 (Dogfish Liver) CRM aliquots were processed with the study samples. For CAAS analysis, nominal 0.0750-g aliquots were transferred to combustion cuvettes and samples were ramped to 800 °C. NIST 2709a (San Joaquin Soil), NRCC TORT-2 (Lobster Hepatopancreas), and NRCC DOLT-3 (Dogfish Liver) reference materials (n = 4) were used to calibrate the CAAS and monitor method performance, respectively.

Soil sample preparation and analysis

The objective of this limited geochemical laboratory analysis was to estimate the overall level of each analyte, not the bioavailable fraction; thus, an acid extraction procedure was employed. After mixing with a plastic spatula, nominal 0.2500-g soil aliquots were transferred to 50-mL plastic tubes, HNO_3 (0.625 mL) and DI H_2O (0.625 mL) were added, and the samples were vortexed. The tubes were ramped to 95 °C and held at temperature for 15 min. Another aliquot (0.625 mL) of HNO_3 was added; the tubes were vortexed, returned to the hot block, ramped to 95 °C, and held for 120 min. H_2O_2 (0.375 mL) and DI H_2O (0.250 mL) were added, the tubes were vortexed and returned to the hot block, and the temperature was ramped 95 °C and held for 30 min. After cooling to room temperature, the samples were brought to a final 12.5 mL volume with DI H_2O and centrifuged for 30 min at 3500 rpm before the supernatant was analyzed. Duplicate aliquots of NIST 2711 (Montana Soil) SRM were processed with the study samples. For CAAS analysis, nominal 0.0400-g soil aliquots were transferred to combustion cuvettes and the samples were ramped to 800 °C. Aliquots of NIST SRM 2702 (Inorganics in Marine Sediment) were used to calibrate the CAAS and monitor method performance (n = 5). Cd,

Table 4 Elemental content in freshwater fish samples collected from CKDu endemic area

Analyte	Determined conc.[a] (mg/kg)		Determined conc.[b] (mg/kg)		Determined conc.[c] (mg/kg)	
	Min.	Max.	Min.	Max.	Min.	Max.
As	0.0791	0.148	NA	NA	60.5	214
Ca	886	16,900	130	248	60.5	214
Cd	0.00035	0.00090	<LOD[d]	0.2	0	0.5
Cr	0.00557	0.0736	NA	NA	NA	NA
Cu	0.0617	0.268	0.48	2.33	0.3	1.2
Fe	2.02	16.3	1.9	8.9	2.2	5.8
Hg	0.00380	0.189	<LOD	0.025	0.002	0.234
K	597	1780	3250	4750	1746	4194
Mg	72.5	210	225	315	235	330
Mn	0.149	9.97	<LOD	0.80	0.1	1.2
Na	312	513	328	460	236	763
Ni	0.0118	0.0884	NA	NA	NA	NA
Pb	0.00542	0.114	NA	NA	NA	NA
Se	0.0573	0.162	NA	NA	NA	NA
Sn	0.00062	0.00988	NA	NA	NA	NA
Zn	3.32	14.2	4.0	5.3	2.9	5.7

NA not applicable; not analyzed

[a] Determined concentration (n = 11 tilapia samples) from this study; dry tissue mass

[b] Determined concentration (n = 14 tilapia; muscle only (Allinson et al. 2009); wet tissue mass)

[c] Determined concentration (n = 17 tilapia; muscle only (Allinson et al. 2002); wet tissue mass)

[d] Determined value reported as less than limit of detection, LOD

Pb, Se, and Sn were determined by ICP-MS, As, Ca, Cu, Cr, Fe, Mg, Mn, Ni, and Zn by ICP-OES, and Hg by CAAS.

Water sample preparation and analysis

For ICP measurements, 20 mL of each sample was transferred to a 50-mL tube for sample preparation. HNO_3 (2.00 mL), HCl (2.00 mL), and 1000 μg/mL Au (0.05 mL) were added; the tubes were heated in a digitally controlled hot block at 95 °C for 2 h and brought to 40 mL with DI H_2O after cooling to room temperature. Triplicate matrix spikes were prepared at nominal 10 ng/mL and analyzed with study samples to assess analyte recovery. For IC measurements, samples were diluted 10-fold with DI H_2O. Using Dionex AS18 analytical and guard columns, baseline resolution of F from interferences (i.e., nitrate from acid stabilization) was achieved. As, Cd, Cu, Cr, Fe, Pb, Mn, Hg, Ni, Se, Sn, and Zn were determined by ICP-MS, Ca, Mg, K, and Na by ICP-OES, and F by IC.

Results and discussion

Method performance

Several laboratory QC samples were prepared and analyzed with each batch of biological and environmental study samples to continuously monitor method performance. For each sample matrix, method blank data consistently indicated that analyte background contribution from the employed reagents and procedures was not significant. Summary data tables from reference materials processed with hair, rice, freshwater fish, and soil samples are presented in supplemental tables S1–S4, respectively.

Blood elemental content

The concentration of a suite elements was determined in whole blood (n = 83) samples. The determined range and mean of each analyte is provided in Table 1. For duplicate aliquots of NIST 955c Caprine Blood (Level 3),

Table 5 Comparison of soil elemental content versus US EPA human soil screening values

Analyte	Determined concentration (mg/kg)			Human SSL[a]	Exceedance
	Min.	Max.	Mean		
As	3.39	11.9	7.32	6	Yes
Ca	278	3780	1990	NA	No
Cd	0.0182	0.190	0.109	70	No
Cr	24.4	118	59.2	0.36[b]	Yes
Cu	6.58	49.6	20.2	3100	No
Fe	8820	43,200	22,700	55,000	No
Hg	0.0161	0.675	0.0722	9.4	No
Mg	841	3020	1830	NA	No
Mn	162	2340	970	1800	Yes
Ni	2.42	35.5	15.3	1,500[b]	No
Pb	2.79	46.4	10.0	400	No
Se	0.672	3.61	1.71	390	No
Sn	0.00799	0.365	0.0611	47,000	No
Zn	7.00	50.9	24.0	23,000	No

NA not applicable; data not available

[a] US EPA Regional Soil Screening Level (SSL) for risk; residential soils (US EPA 2016a, b)

[b] Screening levels based on nickel soluble salts and hexavalent chromium

processed with blood study samples, the average recovery for certified analytes was 127 % (16 % RSD) of the certified level (5.201 μg/L) for Cd and 100 % (1.7 % RSD) of the certified level (277.7 μg/L) for Pb.

Blood Cd levels can confirm a recent acute exposure, but urinary Cd more closely reflects low to moderate chronic exposure over time and the overall body burden (Jarup 2002). Overall, determined whole blood Cd concentrations in many of the study samples (n = 57) exceeded the mean US reference value (CDC 2015) for healthy nonsmokers (0.424 μg/L). The maximum observed concentration of Cd in whole blood (3.11 μg/L) from this study was less than the level that the US Occupational Safety and Health Administration considers hazardous (OSHA 2004), but OSHA standards are not solely health-based, do not take into account sensitive subpopulations, and may assume limited exposure duration (e.g., 8 h/day) and the use of personal protective equipment. These results suggest that periodic monitoring of blood and urinary Cd from at least a subset of residents living within the CKDu endemic area would further characterize whether biological Cd concentrations in this region may act as an environmental nephrotoxin. In the current investigation, determined blood Pb concentrations also exceeded the mean US reference value (1.90 μg/dL) and blood lead level where adverse health effects in both children and adults can occur (5 μg/dL) in 74 and 14 of the 83 study samples, respectively (CDC 2015; NTP 2012; CDC 2012). As there is evidence that Pb exposure at low doses can lead to adverse health effects, including kidney damage, periodic monitoring of blood Pb levels from residents in the CKDu endemic area would also further characterize whether Pb may act as an environmental nephrotoxin for individuals in the NCP region of Sri Lanka.

Hair elemental content

Hair samples (n = 56) were analyzed, and the determined range and mean of each analyte range are provided in Table 2. Because limits of detection (LODs) varied with available mass, samples and values less than LOD were not included in the calculation. Recoveries from the reference material aliquots processed with study samples (Table S1) were within ±25 and ±30 % of certified values for nine and ten of the analytes, respectively, indicating acceptable method performance for the overall panel. The addition of hydrofluoric acid (HF) could improve recoveries for secondary analytes (Cr and Ni) with lower recoveries, but this was beyond the scope of this study. Analyte minimum, maximum, and mean data for all samples (n = 56) are presented in Table 2. Hair is considered to be a preferred biomarker for evaluating Hg exposure over extended periods of time (weeks and months), and a majority of the participants in this study (n = 37) had concentrations exceeding those of women of childbearing age who are frequent fish consumers (geometric mean 0.38 μg/g) in the USA (McDowell et al. 2004).Observed hair Cd concentrations were comparable to those reported in a recent investigation (Jayatilake et al. 2013), but determined As concentrations from this study(n = 51 of 56 samples < LOD of 0.0186 mg/kg) were generally lower than those reported by Jayatilake and colleagues.

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Table 6 Comparison of elemental soil concentrations versus USGS background level

Analyte	Determined concentration[a] (mg/kg)			US background concentration[b] (mg/kg)			Mean background exceedance[c]
	Min.	Max.	Mean	Min.	Max.	Mean	
As	3.39	11.9[b]	7.32[b]	<0.06	1110	6	Yes[c]
Ca	278	3780	1990	<100	330,000	20,000	No
Cd	0.0182	0.190	0.109	<0.1	77	0.3	No
Cr	24.4	118[b]	59.2[b]	<1	4120	36	Yes[c]
Cu	6.58	49.6[b]	20.2[b]	<0.5	5090	18	Yes[c]
Fe	8820	43,200[b]	22,700[b]	<100	140,000	20,000	Yes[c]
Hg	0.0161	0.675[b]	0.0722[b]	<0.01	56	0.05	Yes[c]
Mg	841	3020	1830	<100	140,000	6000	No
Mn	162	2,340[b]	970[b]	<5	7780	612	Yes[c]
Ni	2.42	35.5[b]	15.3	<0.5	2310	18	No[c]
Pb	2.79	46.4[b]	10.0	<0.5	12,400	26	No[c]
Se	0.672[b]	3.61[b]	1.71[b]	<0.2	8	0.3	Yes[c]
Sn	0.00799	0.365	0.0611	<0.1	375	2	No
Zn	7.00	50.9	24.0	<1	11,700	66	No

[a] US background soil concentrations (USGS 2013), Tables 2 and 3

[b] Soil concentration exceeds mean US background soil concentration for a given element

[c] Min., max., and or/mean soil concentration exceeds the US mean background concentration for a given metal. No soil samples exceeded the maximum US background soil concentration. US background soil concentrations were based on samples collected from 0 to 100 cm below the surface

Rice sample analysis

The concentration of a suite of elements was determined in locally grown rice samples (n = 41), with resulting analyte ranges and mean values presented in Table 3. Recoveries from the reference material aliquots processed with study samples (Table S2) were within ±15 % of certified values for all eight analytes, indicating acceptable method performance for the overall panel. The maximum measured Cd rice concentration was 0.117 mg/kg, which is below the maximum level of 0.4 mg/kg recommended by the Codex Alimentarius Commission (FAO/WHO Commission, 2011) and the maximum allowable concentration (MAC) of 0.2 mg/kg for rice cultivated in China (Fang et al. 2014; Huang et al. 2013). However, given the importance of rice to the Sri Lankan diet, it is possible that Cd levels are present at a concentration above a no adverse health effect threshold given the chronic exposure duration, greater exposure frequency, and higher end ingestion rates throughout the average Sri Lankan lifespan. As a result, regular screening of this staple, evaluation of Cd exposure with body weight mass, and additional risk screening are recommended (Bandara et al. 2007).

Fish sample analysis

A data summary from collected fish samples (n = 11) is presented in Table 4. For the elements with certified reference material values, recoveries (Table S3) were within ±15 % of the certified value for 13 of the 14 analytes, with the remaining analyte recovery within ±20 % of the certified value, indicating acceptable method performance for the overall panel. Freshwater, reservoir-caught fish are an important source of protein for Sri Lankans living in the NCP (Allinson et al. 2002). For comparison, data from other recent fish surveys are presented (Allison et al., 2009; Allison et al., 2002). Observed concentrations between these studies were similar with respect to Cd levels and lower than other recent literature reports (Jayatilake et al. 2013; Bandara et al. 2007). Based on the determined element concentrations, it is unlikely that typical fish consumption poses a major risk factor for CKDu. However, it is important to stress that this study's sample size was limited and not directly linked to consumption habits; thus, additional testing is recommended for a multifactorial study.

Table 7 Comparison of water concentrations versus drinking water standards

Analyte	Determined Concentration (μg/L)			Water std.[a]	Exceedance?
	Min.	Max.	Mean		
As	<LOD[b]	1.55	NA	10	No
Ca	3760	161,000	55,400	NA	No
Cd	<LOD	0.168	NA	3	No
Cu	<LOD	43.7	2.85	2000	No
Cr	<LOD	3.33	NA	100[c]	No
F	78.7	2,050[e]	604[e]	600	Yes[e]
Fe	2.34	1,380[e]	112	300[c]	Yes[e]
Hg	NA	NA	NA	6	No
K	347	55,000	2840	NA	No
Mg	145	58,400	20,500	NA	No
Mn	0.130	389.5[e]	45	50[c]	Yes[e]
Na	749	156,000[e]	39,800	50,000	Yes[e]
Ni	<LOD	9.71	1.38	70	No
Pb	<LOD	13.4[e]	0.672	10	Yes[e]
Se	<LOD	1.99	NA	40	No
Sn	<LOD	0.906	NA	NA	No
Zn	0.606	254	12.9	5,000[d]	No

NA not applicable; mean not calculated or data not available

[a] WHO International Drinking Water Guideline values (WHO 2011) obtained from Table A3.3

[b] Determined concentration less than the limit of detection (LOD); As = 1.00 μg/L (n = 90 < LOD), Cd = 0.140 μg/L (n = 96 < LOD), Cu = 0.220 μg/mL (n = 6 < LOD), Cr = 1.70 μg/L (n = 89 < LOD), Ni = 0.140 μg/L (n = 2 < LOD), Pb = 0.0500 μg/L (n = 2 < LOD), Se = 0.210 μg/L (n = 77 < LOD), Sn = 0.200 μg/L (n = 96 < LOD)

[c] US EPA maximum contaminant levels (MCLs) (US EPA 2016b)

[d] US EPA secondary standard (US EPA 2016a)

[e] Water concentration exceeded WHO International Drinking Water Standards, or if no WHO standards were available, US EPA National Primary Drinking Water Regulation MCLs or secondary drinking water standards

Soil sample analysis

Composite surface soil samples (n = 18) were analyzed, and the minimum, maximum, and mean metal concentrations in the study samples were compared to US human soil screening level (SSL) values to identify whether metal concentrations may pose a risk to human health and should undergo additional risk assessment evaluation (Table 5; US EPA 2015). For elements with certified reference material values (Table S4), recoveries from reference material aliquots processed with study samples (Table S4) were within ±15 and ±30 % of certified values for five and eight of the ten analytes with certified concentrations, respectively. Overall, recovery data indicate acceptable performance for the panel.

Screening levels were chosen because they are peer-reviewed, periodically updated, and used for national regulatory-based risk assessments to preliminarily evaluate whether further risk analysis is warranted for specific constituents of concern. The minimum, maximum, and mean metal concentrations in the study samples were also compared to US background soil concentrations to identify whether metal concentrations may be elevated in the study area (Table 6). US soil concentrations were chosen because in 2013, the U.S. Geological Survey (USGS) published a comprehensive report analyzing variation in surface soils throughout the entire contiguous USA, and statistics from this dataset are considered robust in showing variation among different soil types throughout a large region. It does not appear

that such a robust statistical dataset is publicly available in Sri Lanka for comparison purposes. Based on the study results, As, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Se concentrations exceeded mean background US soil concentrations. No study samples exceeded maximum background US soil concentrations. It is important to note that the number of samples from this study is limited, and that additional sampling is recommended in future studies to allow for more robust data evaluation. In addition, an aggressive extraction procedure was employed to solubilize soil metals, rather than a complete digestion or a determination of bioavailability. These results suggest that the presence of mean and maximum concentrations of arsenic, as well as maximum concentrations of manganese, in certain soils located within the NCP could warrant additional study and risk evaluation.

Water sample analysis

Water samples (n = 97) were analyzed, and analyte ranges are provided in Table 7. The average analyte recoveries for triplicate matrix spikes prepared and analyzed with water study samples ranged from 96.3 to 106 % for all of the analytes except for Hg (76.3 %), indicating acceptable method performance. The minimum, maximum, and mean concentrations were compared to applicable drinking water standards to identify whether analyte concentrations may be elevated in the study area. World Health Organization (WHO) International Drinking Water Standards were used for comparison (WHO 2011); if no WHO standards were available, US Environmental Protection Agency (EPA) National Primary Drinking Water Regulations Maximum Contaminant Levels (MCLs) or secondary drinking water standards were used. Based on the study results, F, Fe, Mn, Na, and Pb exceeded applicable drinking water standards in some cases. The endemic CKDu area is known to overlap with a hydrogeochemical region that contains elevated ground F levels and hard water. Elevated F levels in this study were observed, and a large number of samples (n = 73) had "hard" or "very hard" water, as calculated from calcium and magnesium levels only.

Conclusions

The goal of this study was to complete broad panel metallomics and mineralomics analyses of biological (human whole blood and hair), food (rice and freshwater fish), and environmental (soil and drinking water) samples collected from the CKDu endemic area in Sri Lanka's NCP, and use the data from this regional geochemical analysis to inform future multifactorial investigations regarding CKDu risk factors. Element panels were selected for analysis as many literature reports have suggested that fluoride or heavy metals may play a role in the genesis and progression of the disease.

Laboratory analytical findings from the biological study samples are summarized as follows:

- Blood—Determined Cd and Pb blood concentrations exceeded mean US reference values from healthy nonsmokers for 68.7 and 89.2 % of the samples, respectively (CDC 2015).
- Hair—Hg levels in hair exceeded US mean reference values for women of childbearing age in 66.1 % of the samples (McDowell et al. 2004).

These limited blood and hair data suggest that the population within the NCP is subject to chronic, low-level exposure to three nephrotoxic elements—Cd, Pb, and Hg.

Laboratory findings from the food media tested in this study are as follows:

- Rice—The maximum measured Cd rice concentration was below the Codex Alimentarius Commission reference level and MAC for Chinese rice. However, given the importance of rice to the Sri Lankan diet, it is possible that chronic exposure to Cd below available reference levels could act as an environmental nephrotoxin, especially if other nephrotoxins or additional CKDu risk factors are present.
- Fish—Observed concentrations were similar to or lower than other recent literature reports (Jayatilake et al. 2013; Bandara et al. 2007). However, this sample size was limited and not directly linked to consumption habits.

Rice and freshwater fish are both important components of the Sri Lankan diet, and samples obtained from the endemic region indicated that consumption was unlikely to pose a significant health risk from the trace element profile. However, given the relatively small number of samples collected from this limited study and the importance of these dietary food staples,

ongoing monitoring of these foods is recommended, along with initial monitoring of other foods (e.g., vegetables) in any subsequent laboratory analytical analysis designed to capture data that can be used to evaluate potential CKDu risk factors.

Findings from the environmental media tested in this study are as follows:

- Soil—As, Cr, Cu, Fe, Hg, Mn, Ni, Pb, and Se concentrations exceeded mean background US soil concentrations. No study samples exceeded maximum background US soil concentrations. Within the limited number of soil samples, As levels were determined to be above initial risk screening and mean background concentration reference levels, warranting additional investigation and ongoing monitoring.
- Drinking water—F, Fe, Mn, Na, and Pb exceeded applicable drinking water standards in some cases. The endemic CKDu area is also known to overlap with a hydrogeochemical region that contains elevated ground F levels and hard water. Elevated F levels in this study were observed, and many samples had hard or very hard water. Overall, data collected from study drinking water samples reflected the unique hydrogeochemistry of the region, including elevated levels of several elements (e.g., F and Fe) and the prevalence of hard or very hard water.

In future studies, it is also recommended that additional exposure routes and media are considered in subsequent study design (e.g., vegetable consumption and screening, tobacco use, and potential occupational exposures) to create a more robust dataset and more fully consider cumulative exposures.

Current literature suggests the etiology of Sri Lankan CKDu is likely multifactorial, with no single biological or hydrogeochemical parameter directly related to disease genesis and progression. This limited regional geochemical analysis was conducted to inform study design for a potential subsequent multifactorial investigation that will allow for more intensive statistical measurements, risk analysis, and modeling to improve the likelihood of identifying multiple CKDu risk factors. We postulate that exposure to one or more environmental factors, coupled with behavioral or lifestyle conditions and a genetic predisposition by susceptible populations, may ultimately be responsible for CKDu genesis and progression. As noted in Redmon et al. (2014), an approach that includes multimedia sampling and analysis along with geo-referencing allows for more rigorous comparative data analysis, multivariate linear regression modeling, and human health risk assessment that will decrease the overall uncertainty associated with the corresponding results. Additional biological and environmental data, along with demographic and lifestyle information, will create a robust multimedia dataset and allow researchers to more comprehensively and definitively identify CKDu risk factors. Further refining the CKDu case definition, using an early diagnostic technique, and determining which geographic areas and hydrogeochemical parameters to include would also be critical in further differentiating endemic and non-endemic areas in the region. Lastly, efforts should be made to identify causal commonalities between CKDu in Sri Lanka and CKDu hot spots elsewhere in the world.

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