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Environmental Exposure to Arsenic and Chromium in Children is Associated with Kidney Injury Molecule-1

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Abstract

Environmental hazards from natural or anthropological sources are widespread, especially in the north-central region of Mexico. Children represent a susceptible population due to their unique routes of exposure and special vulnerabilities. In this study we evaluated the association of environmental kidney toxicants exposure and kidney injury biomarkers in children living in San Luis Potosi (SLP), Mexico. A cross-sectional study was conducted with 83 children (5-12 years of age) residents of Villa de Reyes, SLP. Exposure to arsenic, cadmium, chromium, fluoride and lead

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DISCLOSURES

No competing financial interests for all authors.

was assessed in urine, blood and drinking water samples. Almost all tap and well water samples had levels of arsenic (81.5%) and fluoride (100%) above the permissible levels recommended by the World Health Organization. Mean urine arsenic (45.6 ppb) and chromium (61.7 ppb) were higher than the biological exposure index, a reference value in occupational settings. Using multivariate adjusted models, we found a dose-dependent association between kidney injury molecule-1 (KIM-1) across chromium exposure tertiles [(T1: reference, T2: 467 pg/mL; T3: 615 pg/mL) (p -trend=0.001)]. Chromium upper tertile was also associated with higher urinary miR-200c (500 copies/ μ L) and miR-423 (189 copies/ μ L). Arsenic upper tertile was also associated with higher urinary KIM-1 (372 pg/mL). Other kidney injury/functional biomarkers such as serum creatinine, glomerular filtration rate, albuminuria, neutrophil gelatinase-associated lipocalin and miR-21 did not show any association with arsenic, chromium or any of the other toxicants evaluated. We conclude that KIM-1 might serve as a sensitive biomarker for environmental exposure risk assessment and kidney toxicity in children.

Keywords

Children; Chromium; Arsenic; KIM-1; miRNAs

1. INTRODUCTION

Worldwide, one-third of disease burden among children is due to preventable environmental risk factors (Landrigan and Etzel, 2014; WHO, 2006^a). Environmental exposures in early life are not only associated with diseases in childhood but they also heavily influence irreversible health effects later in life (Barker, 2004; Boekelheide *et al.*, 2012). Higher, and sometimes unique exposure as well as uptake routes along with immature metabolic and excretory pathways make children particularly susceptible to environmental exposure risks (WHO, 2006^b). Moreover, during maturation, differentiation and growing stages, their organs and systems may be more vulnerable to damage (WHO, 2006^b).

In Mexico, the environmental hazards are complex and widespread. Water supply for about 75% of the total population relies on groundwater abstraction, which is heavily contaminated with arsenic and fluoride, particularly in the north-central region of Mexico (CONAGUA, 2010; Armienta and Segovia, 2008). In San Luis Potosi, a central-north state in Mexico, the risk of exposure to heavy metals such as arsenic, cadmium, chromium and lead is high as a result of mining and industrial activities (Calderon *et al.*, 2001; Carrizales *et al.*, 2006; Dominguez-Cortinas *et al.*, 2013; Trejo-Acevedo *et al.*, 2009). In animal models, these kidney toxicants have been shown to target predominantly the proximal tubule causing reactive oxygen species generation followed by endoplasmic reticulum stress and mitochondrial damage, culminating in cellular necrosis/apoptosis (Barbier *et al.*, 2005; Sabolic, 2006). In humans, chronic exposure to heavy metals and fluoride has been associated with kidney disease (Soderland *et al.*, 2010; Weidemann *et al.*, 2015). However, most of these studies have been conducted in adults that are occupationally exposed and therefore, the effects of environmental exposure in children largely remains uninvestigated. Another limiting factor is the use of in-sensitive and non-specific biomarkers, such as serum creatinine (SCr), for evaluating kidney injury. Non-invasive and sensitive biomarkers such as

kidney injury molecule 1 (KIM-1) and neutrophil gelatinase-associated lipocalin (NGAL) have demonstrated to be not only more sensitive than SCr, but also specific in detecting tubular damage (Vaidya *et al.*, 2008). Yet another class of promising biomarkers are microRNAs (miRNAs) and in particular, miR-21, miR-200c and miR-423 that showed high sensitivity and specificity to distinguish patients with acute kidney injury (AKI) admitted in the intensive care unit as compared to patients without any clinical evidence of kidney injury (Ramachandran *et al.*, 2013).

The use of sensitive and specific biomarkers that associate with environmental kidney toxicants exposure may allow early diagnosis and facilitate prompt intervention strategies that can prevent irreversible health effects in children later in life. Therefore, the aim of this study was to evaluate the association of exposure to environmental kidney toxicants and kidney injury biomarkers in children living in San Luis Potosi, Mexico.

2. MATERIAL AND METHODS

2.1. Description of the study area

The present study was carried out in Villa de Reyes county, a rural population (10,383 inhabitants) in San Luis Potosi, Mexico. The residents of this locality have reported high incidence of chronic kidney disease (CKD) with unknown etiology, especially in young adults. The Mexican Ministry of Health reported that genitourinary diseases are the third leading cause of death in Villa de Reyes, just after accidents and diabetes-mellitus-related outcomes (SEDESOL, 2010). Additionally, ground water represents the main source of water for domestic purposes but remains heavily contaminated with natural occurring elements such as arsenic and fluoride (Ortiz-Pérez *et al.*, 2006). Twenty-five % of the residents in this community live in extreme poverty, 22.6% do not have basic domestic water supply system and 66% lack access to primary health insurance – factors that significantly increases the vulnerability of this population (CONEVAL, 2010).

2.2. Study design and participants

We performed a cross-sectional sampling of children attending two public elementary schools in Villa de Reyes, in June 2014. The study was approved by the institutional review board at the School of Medicine, Universidad Autónoma de San Luis Potosi. Parents were previously informed about the study and provided a written informed consent form prior to children's enrollment. Children participating were among 107 boys and girls (5 to 12 years old) attending first to sixth grade with uninterrupted residency in the study area since birth. Girls with menarche were excluded. Children who reported congenital kidney diseases or urinary tract infections or those taking nonsteroidal anti-inflammatory drugs or antibiotics were also. Data on residency, drinking and cooking water sources and consumption, marine food consumption as well as, parent's occupation, grade of education, smoking and alcohol consumption habits; were gathered at the time of enrollment using a questionnaire. Body weight and height were recorded as well. A single spot urine sample was collected in sterile plastic cups and placed immediately on ice. Aliquots (2 ml) of the urine supernatant were prepared in sterile polypropylene tubes after centrifugation (3000×g, 10 min) and stored at –80°C. A single blood sample of approximately 12 h fasting was drawn in two different

tubes: one for lead analysis (metal-free vacuoliner EDTA) and another tube for serum isolation. Samples were placed on ice immediately after collection, then blood and serum samples were stored at 4 and -20°C , respectively. On March 2015, the water sampling was performed. From 107 original participants, 63 donated both a tap water (domestic water supplies) and bottled water samples ($n=126$). Additionally, samples from tube well water were collected directly from the three local water systems at three different depths ($n=9$): a superficial (1 m), middle (100 m) and deep level (130 m). All tubes containing samples for heavy metals analysis (urine and water) were previously rinsed in 0.1% HCl (v/v).

2.3. Measurements of kidney toxicants

Quantitation of arsenic, cadmium and, chromium in urine and water samples was performed using a PerkinElmer ELAN DRC-II inductively coupled plasma-mass spectrometer (PerkinElmer Waltham, MA). Lead levels in whole blood were quantified by atomic absorption spectrophotometry using graphite furnace using a Perkin-Elmer 3110 atomic absorption spectrophotometer (PerkinElmer Inc., Houston, TX). Quantification of fluoride in urine and water samples was performed using an ion selective electrode as described previously (Cardenas-Gonzalez *et al.*, 2013). All samples were analyzed in duplicates (%CV <10) with a recovery rate above 95%, for all the assays.

2.4. Determination of Kidney injury biomarkers

Urinary KIM-1 and NGAL were measured using micro-bead assays as described previously (Vaidya *et al.*, 2008). Urinary albumin and urine creatinine and SCr were measured using Daytona auto-analyzer (Randox Laboratories, Virginia). All samples were analyzed in duplicates and the intra-assay CV was below 15%.

2.5. RNA isolation and measurement of microRNAs

RNA isolation—200 μL urinary supernatant was used for isolation with the miRNeasy Serum/Plasma Kit from Qiagen (Valencia, CA) according to manufacturer's instructions.

Reverse transcription (RT) and Pre-Amplification—1.5 μL of the eluted RNA was revers transcribed into cDNA using the miScript RTII kit (Qiagen). The prepared cDNA was diluted five-fold, and 5 μL of the diluted cDNA was then pre-amplified with Qiagen's miScript PreAMP kit. The pre-amplified cDNA was diluted five-fold prior to qPCR detection.

qPCR—A SYBR Green-based qPCR was performed with specific primer probes for miR-21, -200c, -423 and a positive RT control miRTC Qiagen), starting with 2 μL diluted and pre-amplified cDNA in a total reaction volume of 10 μL . The thermal profile was as following: activation 15 sec at 95°C ; 40 cycles of annealing/elongation with 15 sec at 94°C , 30 sec at 60°C and 30 sec at 72°C . Finally, a melt curve analysis was included.

miRNA quantification—Concentration of miRNAs in urine (copies/ μL) were derived from standard curves processed in parallel to the samples with the same protocol. Synthetic RNA oligonucleotides corresponding in sequence to the mature miRNAs measured were used to generate the standard curves (Sigma-Aldrich, Haverhill, MA, USA). Serial dilutions covered

the range from 7.35×10^4 to 9 copies/ μ l and all three standard curves had high correlation coefficients ($r=0.98-0.99$).

2.6. Statistical Analysis

We first performed exploratory analyses to assess data quality and consistency and the distribution of the variables of interest. Values below the limit of detection were substituted by LOD/ 2. Continuous variables presented non-normal distribution for all except for chromium, glucose, total cholesterol and LDL. Spearman's correlation coefficient for urinary KIM-1, NGAL, miR-21, miR200c, miR-423, SCr, estimated glomerular filtration rate (eGFR) and albumin-creatinine ratio (ACR) were performed among all the kidney toxicants of interest.

We used linear regression models for kidney injury biomarkers evaluated on a continuous scale and calculated beta-coefficients and 95% CIs. Kidney toxicants were modeled on a continuous scale or categorized according to tertiles of the distribution as follows: arsenic (<28, 29-42.5 and >42.5 ppb), chromium (<46, 47-69, >69 ppb), fluoride (<1.8; 1.8-2.8; >2.8 ppm), and blood lead (<4.9; >4.9-7.0; >7.0 μ g/dl). For multivariate linear regression models, we performed three different models:

- *Model 1:* adjusting by the *a priori* confounders age, sex and z-BMI.
- *Model 2:* adjusting by the *a priori* confounders age, sex and z-BMI and urinary specific gravity as a covariate.
- *Model 3:* adjusting by the *a priori* confounders age, sex and z-BMI and urinary creatinine as a covariate.

Further adjustment by atherogenic index, lipid serum profile or fasting glucose were based on Wald tests with a *p*-value of <0.20, or if their inclusion improved the model fit (based on the change in 10% of R^2 value).

To analyze the biomarker data, KIM-1 and NGAL, were modeled as untransformed continuous variable or as square transformed continuous variable. Because inferences based on square transformed KIM-1 and NGAL were comparable (data not shown), results are reported as untransformed variables. Analysis for validation of the multiple regression with robust weight function was performed for all the analyses. Validation of the multiple regression models was performed using studentized residuals. Model predictions were graphed against standardized residuals to assess heteroscedasticity. We used alpha levels of 5% as the reference value for statistical significance and Stata S.E. version 13.1 for the statistical analyses (StataCorp, College Station, TX).

3. RESULTS

3.1. Characteristics of the Study Population

Of the initial 107 child participants, we excluded 12 with no urine or blood sample and 8 with an incomplete questionnaire. Table 1 summarizes the demographics, socioeconomic status, general health and exposure levels in children residents of Villa de Reyes, SLP, Mexico ($n=83$). The population consisted of 36 (43.4%) females and 47 (56.6%) males.

Mean age was 8.1 years, 59% were <8 years old (range, 5-12). Using standard international age- and sex- specific body mass index (z-BMI) cut points based on guidelines from the Centers for Disease Control and Prevention (CDC), 67% of the children were classified as normal weight, 10% as underweight, 23% as overweight, and 14% as obese. Around 70% of the population reported dependence on groundwater (tap water) for drinking and domestic purposes. Only 4% of the population reported any seafood consumption suggesting that seafood is unlikely to be the source of Arsenic. Socioeconomic status (defined by parental education) was in accordance with the local statistics (SEDESOL, 2010) with nearly 20% of one of the parents having a high-school diploma or higher degree. There were no significant differences between males and females in the plasma and blood parameters studied except for glucose (70.4 mg/dl in males vs 59.7 mg/dl in females; $p=0.0005$) and LDL (113.7 vs 91.4 mg/dl; $p=0.039$). In the urinary sediment (Supplementary Table 1), girls had higher levels of white blood cells than boys (10 vs 5 cells/mm³, $p=0.007$).

3.2. Exposure Assessments

Urine arsenic and chromium were exceedingly high (Table 1), even higher than the current occupational exposure limits for adults (Biological Exposure Index [BEI]). Mean urine arsenic was 45.6 ppb (BEI = 35 ppb; 51 % above the BEI), and 61.7 ppb for chromium (BEI = 30 ppb; 90.4 % above the BEI) (ACGIH, 2004^{a, b}). Comparing these levels with those reported by the National Health and Nutrition Examination Survey (NHANES), all the children had arsenic levels higher than those reported for boys (10.2 ppb) and girls (8.2 ppb) between 6-12 years old, whereas 98% of the samples had chromium levels above 15ppm, the 95th upper percentile reported for all ages (Paschal *et al.*, 1998; Jain, 2015). In 66% of the children the levels of lead in blood exceeded the CDC's reference value > 5 µg/dl). In comparison to BEI limits, urine cadmium and fluoride were found to be at normal ranges. Urine fluoride was significantly higher in males than in females (2.8 vs. 2.1 ppm; $p=0.03$).

In order to investigate the source of exposure, the levels of these toxicants were measured in water samples (tap, well and bottled water). Values from well and tap water samples were practically identical. Almost all tap and well water samples had levels of arsenic (81.5%) and fluoride (100%) above the permissible levels recommended by the World Health Organization (WHO) (10 ppb and 1.5 ppm, respectively) (WHO, 2010). The correlation of arsenic, chromium and fluoride levels between urine and water samples was significant only for fluoride ($r=0.34$; $p=0.005$), suggesting that water is the main source of fluoride exposure. Levels of cadmium and lead were below the limit of detection for all samples. Bottled water samples met the international allowable levels for all the toxicants examined.

3.3. Assessment of Kidney Injury

Kidney function evaluated by routine clinical tests as SCr, eGFR, and ACR were within normal limits (Table 2). The eGFR was correlated with age ($r=0.36$; $p=0.003$) and z-BMI ($r=0.27$; $p=0.012$) and was significantly higher in females (93.4 vs 84.0 ml/min, $p=0.01$). ACR levels were also higher in females (0.74 vs 0.33 mg/g creatinine, $p=0.01$). Median urinary KIM-1 was 304.6 pg/ml and there were no differences in KIM-1 by age, sex or z-BMI. Consistent with other studies (Bennett *et al.*, 2015; Pennemans *et al.*, 2013), urinary NGAL was highly influenced by sex, being higher in females (40.37 vs 3.20 ng/ml; $p=0.0001$).

NGAL was also correlated with z-BMI ($r=0.24$; $p=0.024$). These findings were maintained when the biomarkers were normalized by urinary creatinine. Among the three miRNAs, neither of them showed any correlations with age, sex or z-BMI.

3.4. Univariate associations between environmental exposure and biomarkers of kidney injury

Urinary KIM-1 was correlated with urine arsenic and chromium whereas, NGAL correlated with arsenic (Table 3). Urinary miR-200c was correlated with chromium and fluoride whereas, miR-423 correlated with chromium. There was no correlation between any of the other biomarkers and toxicants exposure levels. High correlations were found between miR-200c and -423 with KIM-1 ($r=0.33$; $p=0.002$ and $r=0.42$; $p=0.0001$, respectively). Because urinary creatinine or specific gravity are traditionally used to normalize urinary kidney injury biomarkers, we specifically evaluated the correlations between these variables. KIM-1 was highly correlated with urinary creatinine ($r=0.56$; $p<0.0001$). There was no correlation between KIM-1 and urinary specific gravity. No correlation was found between NGAL, miR-21 and urinary creatinine or specific gravity. MiR200c and miR-423 were highly correlated with both urine creatinine ($r=0.40$ and 0.41 , respectively; $p<0.0001$ for both) and specific gravity ($r=0.41$ and 0.39 , respectively; $p=0.0001$ for both).

3.5. Multivariate models to identify associations between environmental exposure and kidney injury biomarkers

Tables 4 and 5 show the robust multivariate linear regression models evaluating the association between kidney toxicants exposure (urine arsenic and chromium, entered as tertiles) and kidney injury biomarkers (Figure 1). In model 1, there were significant positive associations between children's chromium exposure and KIM-1, miR-200c and miR-423, and also between arsenic exposure and KIM-1, after controlling for age, sex and, z-BMI (Table 4). Urinary KIM-1 increased across the tertiles of urine chromium. Compared with the first tertile of urine chromium, mean KIM-1 for the second and third tertile were 466.7 and 614.6 pg/ml respectively (p for trend=0.001). Similarly, children in the highest tertile of urine arsenic exposure had higher urinary KIM-1 compared with those in the lowest tertile (372.2 pg/ml; 95% CI: 7.6-736.7; p -value=0.046) (Table 5). In Model 2 where urine specific gravity was treated as a covariate after adjustment by age, sex and z-BMI, only KIM-1 showed significant association with chromium as well as arsenic exposure (Model 2) (Table 4 and 5). In contrast, these associations were no longer significant when urine creatinine was used as a separate covariate instead of urine specific gravity (Model 3) (Table 4 and 5). Urine chromium and arsenic were not associated with NGAL or miR-21 or any of the traditional biomarkers assessed, in any of the three models (Table 6). Although, there was no significant difference in the effect estimates (more than 10% without adjusting for age, sex and z-BMI) using unadjusted models (Supplementary Table 2), we considered the adjusted model more appropriate because variables like age, sex and z-BMI have been consistently employed as cofounders in a wide variety of environmental epidemiological studies.

Regression models examining the association between urine cadmium, fluoride or blood lead and the kidney injury biomarkers did not show any statistically significant differences (data not shown).

3.6. Sensitivity Analysis

The inclusion of other variables related to general health status (hematic biometry and blood chemistry) did not significantly change the results of the models (data not shown). The results of the regression analyses were consistent irrespective of whether KIM-1, NGAL and the miRNAs were log-transformed or whether they were treated as non-transformed variables. Regression analysis assessing interactions between kidney toxicants were not significant. Models presented were run without outliers giving the same direction and meaning. The data did not show evidence of co-linearity, as variance inflation factors for all regressions ranged between 1.22 and 1.56.

4. DISCUSSION

A consensus exists that cumulative lifetime environmental exposure in conjunction with other comorbid conditions, accelerates the rate of deterioration in kidney function, increasing the risk for kidney diseases (Kataria *et al.*, 2015; Weidemann, Weaver and Fadrowski, 2015). In Mexico, the environmental hazards from natural or anthropological sources are widespread. Additionally, diabetes mellitus, hypertension and obesity are the most common chronic illnesses in the Mexican population (ENSANUT, 2012) and have been recognized as the leading risk factors for CKD (Jha *et al.*, 2013). Children are a vulnerable group but also represent a population free of significant comorbidities, allowing a clearer assessment of the effect of environmental exposure in the kidney. Here we report three novel findings: 1) Children living in Villa de Reyes, San Luis Potosi, Mexico have exceedingly high arsenic and chromium exposure that even exceed the permissible limits for occupational exposure in adults; 2) Arsenic and chromium exposure were significantly associated with higher urinary KIM-1 excretion; 3) Traditional kidney injury/function biomarkers such as SCr, eGFR, ACR along with novel biomarkers like NGAL, miR-21 did not show any association with arsenic, chromium or any other of the toxicants evaluated. To the best of our knowledge, this is the first study reporting the utility of urinary KIM-1 as a biomarker to evaluate kidney tubular injury induced by environmental exposure in children.

Chromium and arsenic are widespread in the environment. Important sources of exposure to chromium are air, soil, water, food and tobacco products (ATSDR, 2012). Naturally occurring chromium compounds are generally in the trivalent state, while hexavalent chromium compounds (the most toxic form) are mostly derived from human activities (ATSDR, 2012). In the present study, water was not a relevant source for chromium exposure. The evaluation of potential sources for chromium exposure, such as air and soil, are urgently needed in this community.

Drinking water represents the main source of human exposure to arsenic (ATSDR, 2007). In the present study we found significantly high exposure to arsenic, where drinking water represented a partial source of arsenic exposure. Food also represents an important source of arsenic, particularly for organic arsenic compounds (Jackson *et al.*, 2012). Seafood is not a likely source of arsenic in this community as only 4% of the participants reported any consumption of seafood. However, food prepared in arsenic-containing water could represent an important source of arsenic exposure. Almost all participants (95 %) use non-bottled water to cook broths, soups, and beans. This practice can increase the content of

arsenic by 10–30% for most foods, and by 200–250% for beans and grains, which absorb cooking water (Mead, 2005).

In animal models, the proximal tubule is the presumed target of action of these metals, where they induce oxidative stress followed by lipid peroxidation, apoptosis and necrosis (Barbier *et al.*, 2005; Sabolic, 2006). Few epidemiological studies have specifically assessed chromium and arsenic exposure as potential risk factors for kidney disease (Wedeen and Qian, 1991; Zheng *et al.*, 2014). The lack of sufficient evidence in humans is principally due to the lack of study quality and prospective evidence, together with the use of non-sensitive kidney injury biomarkers.

KIM-1 is a transmembrane glycoprotein expressed by the kidney proximal tubular epithelial cells early after injury, followed by cleavage of its ectodomain into the urine (Ichimura *et al.*, 1998; Ichimura *et al.*, 2004; Vaidya *et al.*, 2010). KIM-1 is involved in kidney repair after AKI, promoting the clearance of apoptotic cells (Ichimura *et al.*, 2008). Chronic KIM-1 induction however, has been shown to be maladaptive and promotes fibrosis (Ferenbach and Bonventre, 2015) and this correlates with the persistent induction and excretion of KIM-1 in variety of inflammatory and fibrotic pathogenesis in the kidney (van Timmeren *et al.*, 2007). In environmental settings, KIM-1 has demonstrated to be more sensitive than N-acetyl- β -D-glucosaminidase and β 2 microglobulin for the detection of kidney injury induced by chronic cadmium exposure (Prozialeck *et al.*, 2007; Ruangyuttikarn *et al.*, 2013). We found that KIM-1, among all the biomarkers examined, was most strongly associated with chromium and arsenic exposure. Therefore, we suggest that urinary KIM-1 might be a promising biomarker for kidney injury risk assessment in populations exposed to environmental toxicants.

NGAL is a member of the lipocalin family proteins expressed and secreted by immune cells, hepatocytes and kidney tubular cells (Devarajan, 2010). Elevated urine NGAL has been shown to be an early biomarker of AKI and CKD progression in adults and children (Bolignano *et al.*, 2009). Additionally, NGAL was significantly elevated after exposure to PM_{2.5} in welding workers (Chuang *et al.*, 2015). In children, elevated urine NGAL was found in patients with common pediatric kidney diseases with decreased GFR (Nishida *et al.*, 2010). In this study, we found higher urinary NGAL in girls than in boys, confirming previous reports in other pediatric populations (Bennett *et al.*, 2015; Pennemans *et al.*, 2013). Pyuria is an important potential confounder for NGAL measurements (Schinstock, 2013); we observed significantly higher counts of white blood cells in the urinary sediments of girls than boys. It is possible that higher levels of NGAL in the girls were induced by post-renal causes, like urinary tract infections, rather than exposure to kidney toxicants. MiRNAs might be sensitive biomarkers for environmental exposure and effect (Vrijens *et al.*, 2015). Moreover, they could provide information about pathways perturbation in the kidney that may aid in understanding the pathogenesis of kidney injury initiated by environmental exposure. We selected miR-21, -200c and -423, which were previously identified as sensitive biomarkers for kidney injury in adults (Ramachandran *et al.*, 2013). We found that only high chromium exposure levels were associated with miR-200c and miR-423. The lack of associations with miR-21 or stronger associations with the other miRNAs could be due to the differences in age, type of kidney injury and response between study populations. The

conventional markers of measuring kidney function - GFR, ACR, and SCr did not show any association with any of the kidney toxicant exposures potentially due to the renal reserve capacity that renders these markers insensitive and delayed (Bosch, 1995).

Urine dilution adjustment has been one of the biggest challenges in environmental epidemiology. Although, adjustment by urinary creatinine is traditionally used, Weaver et al. (Weaver *et al.*, 2016) have shown that this could potentially lead to inconsistencies because of a misleading statistical effect, by which the associations between urine creatinine-adjusted toxicants and kidney outcomes may be statistical rather than biological. Other approaches to account for urine dilution include timed urine collections, usually over a 24 h period (Waikar *et al.*, 2010), overnight spot urine sample (first morning) (Akerstrom *et al.*, 2014), adjustments using urine specific gravity (Suwazono *et al.*, 2005), using urine creatinine as a covariant in the regression model (Barr *et al.*, 2005) or not adjusting at all (Ikeda *et al.*, 2003; Moriguchi *et al.*, 2003). In the present study, we used three different methods to deal with this issue: Model 1 was an unadjusted model, whereas Model 2 and 3 used urinary specific gravity or creatinine, respectively as separate covariates. However, an important characteristic of any covariant is that it cannot be downstream to the outcome in the causal pathway (Greenland and Morgenstern, 2001). Urinary creatinine and specific gravity may be affected in advanced injury stages or after long-term exposure periods, just after KIM-1 (early outcome). Thus, adding them as separate covariates in the regression models could induce non-causal associations and inconsistent results. Based on this, we considered that Model 1 best reflected the association between environmental exposure and kidney injury (Figure 1).

The main strength of this study is the comprehensive nature of the associations between exposures to 5 different environmental contaminants with 8 biomarkers of kidney injury. Assessment of traditional biomarkers of kidney dysfunction along with novel biomarkers such as KIM-1 and candidate miRNAs in response to environmental exposure in children has never been performed before. Some of the limitations of this study include a potential reverse causality because the metal contaminants were measured in the urine as opposed to being measured in the blood/toe-nail/hair samples. Yet another limitation is that in our study we measured total arsenic and chromium without specifically measuring the non-toxic form (As^{5+} or Cr^{3+}) and the toxic form (As^{3+} or Cr^{6+}). Speciation data would provide more accurate risk assessment, improving strategies to prevent exposure and adverse health effects. Additional limitations are the sample size that limited the power of our analysis and the lack of well-established reference values for all of the novel biomarkers that makes our data difficult to interpret. Finally, due to the cross-sectional nature of our study design, we cannot establish a valid causal relationship between exposure and outcomes.

In summary, our results demonstrated a strong association between chromium exposure and urinary KIM-1. Further, high levels of chromium were associated with high urinary miR-200c and miR-423. We also found an association between high levels of arsenic exposure and higher urinary KIM-1. Although these children are asymptomatic in the clinical settings, based on the increases in KIM-1 it is likely that kidney injury initiates at these early stages, potentially increasing their susceptibility to kidney disease later in life.

Based on our findings we conclude that KIM-1 might serve as an early and sensitive biomarker for environmental exposure risk assessment and kidney toxicity in children.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Highlights

- Children living in Mexico had exceedingly high arsenic and chromium exposure
- Arsenic and chromium exposure was significantly associated with urinary KIM-1
- KIM-1 might serve as a sensitive biomarker to evaluate kidney injury in children

Figure 1.

The relationship between children's urine chromium and arsenic exposure levels (ppb) in tertiles and novel and traditional kidney injury biomarkers. Results are presented as adjusted predictions of KIM-1, NGAL, miR-21, miR-200c, miR-423, SCr, eGFR and ACR with 95% CIs. The associations were adjusted for age, sex, z-BMI (Model 1). Red circles indicate the adjusted means of a specific kidney injury biomarker for each tertile of urine chromium (9.2-46 ppb; 47-69 ppb; 70-123.6 ppb), whereas blue circles indicate adjusted means of a

specific kidney injury biomarker for each tertile of urine arsenic (12-28.3 ppb; 29-42.5 ppb; 43-254 ppb). * $p<0.05$ and ** $p<0.005$.

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Table 1

Characteristics of the Study Population in Villa de Reyes, SLP, Mexico.

a) Demographic and socioeconomic characteristics (n=83)	Mean ± SD or n (%).
Age (y)	8.13 ± 1.93
Male	47 (56.63)
BMI z-score (percentile)	51.93 ± 34.5
Drinking tap Water	61 (73.5)
Parental Educational Attainment (>16 y) ^a	16 (19.3)
b) Plasma and blood analysis (n=81)	GM (95% CI)
Glucose (mg/dl)	64.21 (61.19 to 67.39)
Triglycerides (mg/dl)	96.41 (89.04 to 104.39)
Total cholesterol (mg/dl)	179.35 (169.72 to 189.53)
HDL cholesterol (mg/dl)	61.70 (59.11 to 64.40)
LDL cholesterol (mg/dl)	89.53 (77.85 to 102.97)
VLDL cholesterol (mg/dl)	19.28 (17.81 to 20.88)
Atherogenic index	2.92 (2.72 to 3.13)
Hemoglobin (g/dl)	13.72 (13.52 to 13.93)
Hematocrit (%)	38.46 (37.82 to 39.10)
d) Exposure assessment (Urine and blood, n=83)	GM (range)
Arsenic (ppb)	37.36 (11.91-254.63)
Cadmium (ppb)	0.02 (0.007-0.80)
Chromium (ppb)	56.17 (9.17-123.61)
Fluoride (ppm)	2.18 (0.34-8.60)
Blood Lead (µg/dl)	5.95 (1.47-26.89)
d) Exposure assessment (Tap-water, n=63)	GM (range)
Arsenic (ppb)	12.13 (7.56-16.44)
Cadmium (ppb)	<LOD
Chromium (ppb)	9.39 (6.0-13.03)
Fluoride (ppm)	2.47 (2.08-2.94)
Lead (ppb)	<LOD

Abbreviations: SD, Standard Deviation; GM, Geometric mean; CI, confidence interval; BMI, Body Mass Index ; LOD, Limit of detection.

Note: All metal determinations are referred as total metal concentration.

LOD= 0.04, 0.01, 0.06, 0.007 ppb and 0.02 ppm for arsenic, cadmium, chromium, lead and fluoride, respectively, and 1ppm, for lead in blood.

Reference levels in urine: BEI= 35 for arsenic, 5 for cadmium, 30 ppb for chromium and 3 ppm for fluoride. BEI for lead in the blood is 5 µg/dl.

Reference levels in water): 10 ppm for arsenic, 8ppm for cadmium, 50 ppm for chromium, 15 ppb for lead and 1.5 ppm for fluoride.

Bold: values above reference levels.

Table 2

Evaluation of Traditional and Novel Kidney Injury Biomarkers (n=83)

Kidney Biomarker	Mean $\pm$ SD	GM (95% CI)
KIM-1 (pg/ml)	626.59 $\pm$ 620.45	304.63 (216.79 to 428.06)
KIM-1 (pg/mg creatinine)	518.78 $\pm$ 447.60	296.75 (219.16 to 401.81)
NGAL (ng/ml)		
<i>Boys</i>	9.92 $\pm$ 14.40	3.20 (1.99 to 5.13)
<i>Girls</i>	143.10 $\pm$ 222.71	40.37 (21.50 to 75.79)
NGAL (ng/mg creatinine)		
<i>Boys</i>	9.65 $\pm$ 15.26	2.81 (1.71 to 4.62)
<i>Girls</i>	179.75 $\pm$ 308.75	44.98 (23.70 to 85.40)
miR-21 (copies/ μ l)	376.58 $\pm$ 1426.62	102.51 (75.0 to 140.10)
miR-200c (copies/ μ l)	656.05 $\pm$ 1766.29	158.06 (110.59 to 225.89)
miR-423 (copies/ μ l)	277.85 $\pm$ 605.23	121.09 (91.82 to 156.70)
SCr (mg/dl)	0.62 $\pm$ 0.094	0.61 (0.59 to 0.63)
eGFR (mL/min)	88.19 $\pm$ 18.56	86.59 (83.17 to 98.16)
ACR (mg/g creatinine)	1.99 $\pm$ 4.81	0.47 (0.32 to 0.69)

Abbreviations: SD, Standard Deviation; GM, Geometric mean; CI, confidence interval; KIM-1, Kidney Injury Molecule; NGAL, Neutrophil Gelatinase-Associated Lipocalin; SCr, Serum Creatinine; eGFR, estimated Glomerular Filtration Rate; AUC, Albumin-Creatinine Ratio. LOD= 4.4 pg/ml, 0.5 ng/ml and 0.01 mg/l for KIM-1, NGAL and Albumin, respectively.

Bold: *p* value <0.05.

Associations between Kidney Injury Biomarkers and Environmental Kidney Toxicants (n=83).

Table 3

	KIM-1 (pg/ml)	NGAL (ng/ml)	miR-21 (copies/μl)	miR-200c (copies/μl)	miR-423 (copies/μl)	SCr (mg/dl)	eGFR (ml/min)	ACR (mg/gCr)								
	<i>r_s</i>	<i>ρ</i>	<i>r_s</i>	<i>ρ</i>	<i>r_s</i>	<i>ρ</i>	<i>r_s</i>	<i>ρ</i>								
Arsenic (ppb)	0.24	0.03	0.26	0.02	0.17	0.12	0.09	0.37	0.18	0.09	0.09	0.4	-0.01	0.89	-0.01	0.88
Cadmium (ppb)	-0.06	0.61	-0.05	0.63	0.008	0.94	0.14	0.20	0.18	0.11	0.002	0.98	-0.11	0.3	-0.16	0.14
Chromium (ppb)	0.4	0.0002	-0.06	0.58	0.16	0.15	0.35	0.001	0.35	0.001	0.02	0.82	-0.21	0.06	0.01	0.88
Fluoride (ppm)	0.09	0.38	-0.2	0.07	0.05	0.67	0.27	0.01	0.14	0.22	0.07	0.53	-0.19	0.07	0.08	0.45
Blood Lead (μg/dl)	-0.09	0.39	-0.12	0.26	-0.002	0.98	-0.02	0.82	-0.08	0.48	-0.07	0.54	-0.09	0.42	-0.01	0.92

Abbreviations: KIM-1, Kidney Injury Molecule; NGAL, Neutrophil Gelatinase-Associated Lipocalin; SCr, Serum Creatinine; eGFR, estimated Glomerular Filtration Rate; ACR, Albumin-Creatinine Ratio.
Note: For arsenic, cadmium, chromium and fluoride urine levels were used for the correlation analysis.
 Bold: *p* value <0.05.

Table 4

Robust Multivariable-adjusted Linear Regression Analysis of the Associations Between Children's Urine Chromium Exposure Levels (ppb) in Tertiles and Novel Kidney Injury Biomarkers (n=83).

Biomarker	Cr (ppb)	Model 1 (Adjusted by age, sex, zBMI) ^a			Model 2 (Adjusted by age, sex, zBMI, s.g.) ^a			Model 3 (Adjusted by age, sex, zBMI, UCr) ^a		
		β	(95% CI)	p-value	β	(95% CI)	p-value	β	(95% CI)	p-value
KIM-1 (pg/ml)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	466.71 (66.24 to 867.17)		0.023	459.09 (58.52 to 859.66)		0.025	238.91 (-161.65 to 639.46)		0.239
	T3	614.58 (277.48 to 951.68)		0.001	608.18 (272.97 to 943.39)		0.001	118.99 (-293.63 to 531.62)		0.567
NGAL (ng/ml)										
Boys	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	-4.14 (-15.41 to 7.12)		0.462	-0.79 (-11.74 to 10.14)		0.883	-3.09 (-17.81 to 11.64)		0.674
	T3	-8.43 (-20.35 to 3.49)		0.161	-8.32 (-19.79 to 3.15)		0.151	-6.71 (-23.94 to 10.51)		0.436
Girls	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	141.05 (-38.29 to 320.40)		0.119	140.70 (-52.44 to 333.84)		0.147	151.21 (-28.64 to 331.06)		0.096
	T3	-18.69 (-160.23 to 122.85)		0.789	-18.49 (-158.84 to 121.86)		0.790	14.91 (-134.85 to 164.67)		0.840
miR-21 (copies/μl)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	667.87 (-587.74 to 1923.49)		0.293	473.13 (-523.86 to 1470.12)		0.348	322.84 (-388.72 to 1034.42)		0.369
	T3	118.11 (-177.90 to 414.13)		0.429	-276.75 (-886.71 to 333.20)		0.369	-632.50 (-1753.68 to 488.66)		0.265
miR-200c (copies/μl)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	1185.24 (-245.32 to 2615.78)		0.103	847.74 (-298.77 to 1994.25)		0.145	560.16 (-262.31 to 1382.64)		0.179
	T3	499.45 (56.05 to 942.85)		0.028	-184.85 (-995.16 to 625.46)		0.651	-860.40 (-2166.50 to 445.69)		0.193
miR-423 (copies/μl)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	378.94 (-122.03 to 879.71)		0.136	261.64 (-142.58 to 665.87)		0.201	195.52 (-92.34 to 483.38)		0.180
	T3	189.34 (22.87 to 348.16)		0.026	-52.26 (-351.16 to 246.64)		0.729	-213.44 (-663.85 to 236.95)		0.348

Abbreviations: z-BMI, Body Mass Index z-score; s.g., specific gravity; UCr, Urine Creatinine.

Tertile ranges for Chromium (Cr): T1 (n=28)= 9.2-46 ppb; T2 (n=28)= 47-69 ppb; T3 (n=27)= 70-123.6 ppb.

Bold: p value <0.05.

For NGAL sex was not included in the models.

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Robust Multivariable-adjusted Linear Regression Analysis of the Associations Between Children's Urine Arsenic Exposure Levels (ppb) in Tertiles and Novel Kidney Injury Biomarkers (n=83).

Table 5

Biomarker	As (ppb)	Model 1 (Adjusted by age, sex, zBMI) ^a			Model 2 (Adjusted by age, sex, zBMI, s.g.) ^a			Model 3 (Adjusted by age, sex, zBMI, UCr) ^a		
		β (95% CI)	p-value		β (95% CI)	p-value		β (95% CI)	p-value	
KIM-1 (pg/ml)	T1	0 (reference)	-		0 (reference)	-		0 (reference)	-	
	T2	182.84 (-107.27 to 472.94)	0.213		172.50 (-123.26 to 468.26)	0.249		45.32 (-212.99 to 303.65)	0.728	
	T3	372.16 (7.63 to 736.70)	0.046		362.68(3.12 to 722.25)	0.048		102.23 (-180.67 to 385.12)	0.474	
NGAL (ng/ml)										
Boys	T1	0 (reference)	-		0 (reference)	-		0 (reference)	-	
	T2	0.35 (-9.48 to 10.17)	0.943		1.67 (-8.81 to 12.15)	0.749		1.40 (-8.36 to 11.17)	0.773	
	T3	4.65 (-5.41 to 14.71)	0.356		6.18 (-4.59 to 16.95)	0.253		7.79 (-3.63 to 19.20)	0.176	
Girls	T1	0 (reference)	-		0 (reference)	-		0 (reference)	-	
	T2	-47.80 (-210.33 to 114.73)	0.553		-46.47 (-211.87 to 118.94)	0.570		151.21 (-28.64 to 331.06)	0.096	
	T3	7.38 (-181.85 to 196.63)	0.937		14.43 (-178.23 to 207.08)	0.879		14.91 (-134.85 to 164.67)	0.840	
miR-21 (copies/μl)	T1	0 (reference)			0 (reference)			0 (reference)		
	T2	21.18 (-151.33 to 193.69)	0.807		3.15 (-181.87 to 188.17)	0.973		-84.38 (-377.65 to 208.89)	0.568	
	T3	551.17 (-281.08 to 1383.42)	0.191		488.76 (-260.65 to 1238.18)	0.198		343.94 (-171.62 to 859.51)	0.188	
miR-200c (copies/μl)	T1	0 (reference)			0 (reference)			0 (reference)		
	T2	33.52 (-352.69 to 419.74)	0.863		-12.25 (-394.26 to 369.75)	0.949		-199.32 (-662.62 to 263.98)	0.394	
	T3	929.86 (-179.87 to 2039.59)	0.099		771.45 (-257.85 to 1800.75)	0.140		472.79 (-310.89 to 1256.47)	0.233	
miR-423 (copies/μl)	T1	0 (reference)			0 (reference)			0 (reference)		
	T2	26.78 (-107.61 to 161.18)	0.693		9.78 (-121.59 to 141.15)	0.883		-44.04 (-201.21 to 113.13)	0.578	
	T3	303.97 (-45.56 to 653.50)	0.087		245.13 (-70.17 to 560.43)	0.126		164.93 (-69.09 to 398.97)	0.165	

Abbreviations: z-BMI, Body Mass Index z-score; s.g, specific gravity; UCr, Urine Creatinine.

Tertile ranges for Arsenic (As): T1 (n=28)= 12-28.3 ppb; T2 (n=28)= 29-42.5 ppb; T3 (n=27)= 43-254 ppb.

Bold: p value <0.05.

β For NGAL sex was not include in the models.

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Table 6

Robust Multivariable-adjusted Linear Regression Analysis of the Associations Between Children's Urine Arsenic and Chromium Exposure Levels (ppb) in Tertiles and Traditional Kidney Injury Biomarkers (n=83).

Biomarker	Cr (ppb)	Model 1 (Adjusted by age, sex, zBMI)			Model 2 (Adjusted by age, sex, zBMI, s.g.)			Model 3 (Adjusted by age, sex, zBMI, UCr)		
		β	(95% CI)	p-value	β	(95% CI)	p-value	β	(95% CI)	p-value
SCr (mg/dl)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	0.006 (-0.04 to 0.05)		0.800	1.79 (-1.12 to 4.70)		0.224	1.55 (-1.11 to 4.21)		0.250
	T3	0.001 (-0.06 to 0.06)		0.965	2.78 (-0.99 to 6.53)		0.147	2.31 (-1.24 to 5.86)		0.199
eGFR (ml/min)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	3.17 (-4.39 to 10.73)		0.406	2.97 (-4.50 to 10.45)		0.431	4.58 (-3.27 to 12.43)		0.248
	T3	3.81 (-7.59 to 15.21)		0.508	3.64 (-7.77 to 15.06)		0.527	6.89 (-6.14 to 19.92)		0.296
ACR (mg/gCr)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	1.31 (-1.13 to 3.75)		0.290	1.79 (-1.12 to 4.70)		0.224	1.55 (-1.11 to 4.21)		0.250
	T3	1.79 (-1.72 to 5.30)		0.314	2.78 (-0.99 to 6.53)		0.147	2.31 (-1.24 to 5.86)		0.199
As (ppb)										
SCr (mg/dl)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	0.02 (-0.03 to 0.06)		0.446	0.02 (-0.03 to 0.06)		0.414	0.01 (-0.03 to 0.06)		0.511
	T3	0.001 (-0.05 to 0.05)		0.963	0.002 (-0.05 to 0.05)		0.930	-0.004 (-0.05 to 0.05)		0.890
eGFR (ml/min)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	-3.58 (-10.85 to 3.68)		0.329	-3.85 (-11.11 to 3.40)		0.293	-3.38 (-10.65 to 3.89)		0.358
	T3	1.77 (-7.08 to 10.62)		0.692	1.52 (-7.41 to 10.45)		0.736	2.17 (-7.99 to 12.33)		0.672
ACR (mg/gCr)	T1	0 (reference)		-	0 (reference)		-	0 (reference)		-
	T2	-0.99 (-2.83 to 0.85)		0.286	-1.01 (-2.86 to 0.84)		0.280	-1.11 (-3.09 to 0.86)		0.263
	T3	-0.003 (-2.87 to 2.87)		0.998	-0.06 (-2.59 to 2.48)		0.964	-0.23 (-2.80 to 2.33)		0.855

Abbreviations: z-BMI, Body Mass Index z-score; s.g., specific gravity; UCr, Urine Creatinine; SCr, Serum Creatinine; eGFR, Glomerular Filtration Rate; ACR, Albumin Creatinine Ratio.

Bold: p value <0.05.