BIOMARKERS OF EARLY KIDNEY DAMAGE IN NICARAGUAN ADOLESCENTS SEPTEMBER-NOVEMBER 2011

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I. INTRODUCTION

From September to November 2011, the Boston University team conducted an investigation of biological markers of early kidney injury among adolescents in 3 departments of Nicaragua. Students were recruited from secondary schools in the municipalities of Chichigalpa (2 schools), Masaya (1 school) and Jinotega (1 school).

We undertook this study because many cases of chronic kidney disease (CKD) have been diagnosed among adults in Nicaragua between the ages of 20 and 30. Although one focus of research into the cause has been on occupational related etiologies, given the usual time period it takes for the kidneys to become sufficiently injured to result in clinically apparent CKD, the early age of onset observed in Nicaragua raises the possibility that renal damage might have already started in childhood. A range of theories have been suggested for early initiation of damage, including direct or indirect (due to contaminants brought home on parents' clothing) occupational exposure during childhood, environmental exposures, infectious diseases, low birth weight, and genetic susceptibility. The purpose of this study was to determine whether there was any evidence of early kidney injury among adolescents (age 12-18) who had not yet entered the workforce. We chose adolescents rather than younger children because they would be more likely to manifest detectable kidney damage. The specific objectives of the investigation were:

> 1) To assess whether there is evidence of early-stage kidney damage among adolescents in different areas of Nicaragua chosen to represent a range of demographic and environmental conditions and, hence, potential risk for kidney damage; and
> 2) To assess whether there are differences in the prevalence of early-stage kidney damage by sex, age, and school that are consistent with the patterns of the CKD epidemic among adults.

It should be emphasized that this investigation was focused on early, subclinical kidney injury and not chronic kidney disease (CKD) itself. The former could be accomplished with urine samples, the collection of which involves no discomfort or risk to participants, while the latter would require blood samples. We decided against the collection of blood samples due to ethical concerns and because this was our first exploratory study with children at Nicaraguan schools. We also had no reason to suspect that kidney damage in the students, if present, would have progressed to actual CKD.

II. METHODS

Study protocols were reviewed and approved by the Institutional Review Boards at the Boston University Medical Center, the Nicaraguan Ministry of Health, and Universidad Autónoma de Madrid. As the study population was mainly younger than 18 years old, the parents of all study

subjects provided written informed consent and adolescents were asked to provide assent prior to participation in the research activities.

2.1 Study population and design

The study population included students in four schools, which were selected based on *a priori* hypotheses to represent a range of demographic and environmental conditions and, hence, potential risks for kidney damage. Ranked from lowest to highest hypothesized risk, the schools were:

1. School 1 in the city of Jinotega in the department of Jinotega, an area located at 800 meters above sea level with many coffee plantations. Areas at higher altitude and workers at coffee plantations have been found to have a lower prevalence of CKD in several studies. The mortality rate from CKD in the department of Jinotega was 5 per 100,000 residents in 2010, which was among the lowest rates of any department.

2. School 2 in the city of Masaya in the department of Masaya, located at 220 meters above sea level and an area characterized by small farms and predominantly artisan-related occupations. Masaya is located in the Pacific region, where the excess cases of CKD appear to have been occurring throughout Central America. In 2010, the department of Masaya had a mortality rate from CKD of 12 per 100,000 residents, which was the median rate by department in Nicaragua.

3. School 3 in the northern neighborhood of the city of Chichigalpa in the department of Chinandega, an area in the Pacific region located almost at sea level, and characterized by production of crops such as sugar cane, rice, and peanuts. The department of Chinandega had a mortality rate from CKD of 95 per 100,000 residents, the second highest rate of any department in Nicaragua in 2010.

4. School 4 in the southern neighborhood of the city of Chichigalpa, where almost all parents currently or in the past have worked in the sugar cane industry, and where an earlier prevalence study conducted by a different group of investigators had found high rates of CKD (Torres, 2008).

To be eligible for participation in this study, students were required to be 12-18 years old and have never worked in a manual labor job, as our goal was to assess kidney damage prior to occupational exposures. Prior work experience was defined as at least one month of manual labor, whether paid or done to help the family. Students who had already participated in sports or vigorous exercise on the same day as samples were being collected, and female students who were currently menstruating were also excluded. At School 4 (southern Chichigalpa), we aimed

to only select children of a parent who currently or formerly worked in the sugar cane industry. At the remaining schools, we excluded children of a parent who ever worked in the sugar cane industry.

Our goal was to collect samples from at least 50 students at each school, with an equal distribution of older (16-18 years) males, older females, younger (12-15 years) males, and younger females.

2.2. Recruitment and enrollment

The recruitment process was planned in coordination with the Ministry of Education's (MOE) professors, including the head of the MOE at the Department and municipal levels. The support coming from MOE Directors at the municipal and school level was a key and important factor for the overall process. The field team spent a full week at each school. Information regarding the purpose, procedures, and inclusion and exclusion criteria was briefly described to students in their classrooms. A packet of material containing a letter of invitation to the study, a parental consent and permission form, and a brief questionnaire was sent home with interested students. After review of the invitation letter, interested parents returned the signed consent form the following day, together with a parental questionnaire, which solicited information on general family characteristics and eligibility criteria (i.e., child's age and parents' occupations; see Appendix 1).

We selected all students who met eligibility criteria based on the parental questionnaire. Study staff then asked the children to assent to participate. Those children who signed the assent form were then asked to complete a questionnaire (see Appendix 2), which was reviewed to assess final eligibility (student's past work history and prior participation in sports or vigorous exercise on that day). Current menstruation status was obtained verbally by a female study staff member. To achieve the desired sex and age distribution, we had to return to certain schools to obtain samples from some girls once their menstrual period was over. A flow chart of the study population at different stages is shown in Figure 1.

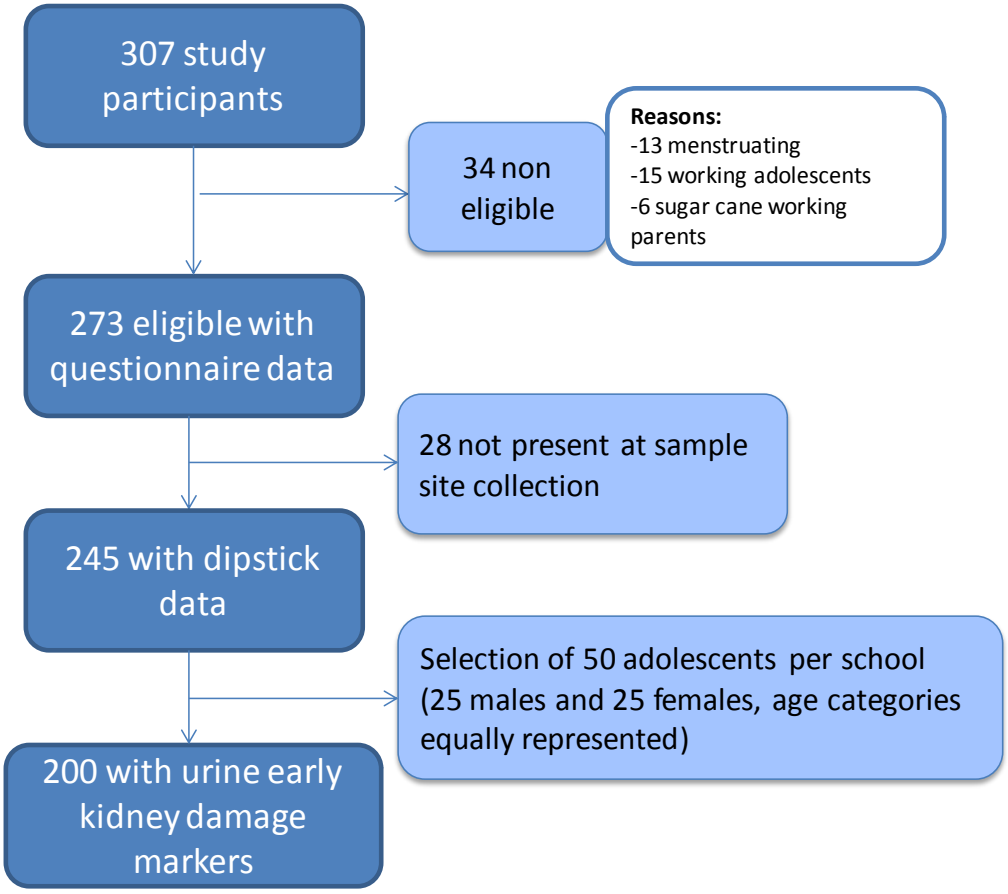
2.3 Collection and analysis of urine samples

2.3.1 Collection of urine samples

Samples at all schools were collected during morning time before classes started to increase the likelihood that all urine samples would be taken before students became physically active. Classes at School 4 usually take place during the afternoon, but we were able to have the students come in during the morning. Our field study personnel, consisting of 2 medical doctors, a nurse and 2 laboratory technicians, obtained the urine samples at the school site from 8-10 a.m. A nurse from the Ministry of Health also supervised the field activities and visited the schools.

Prior to providing urine samples, participants were asked to wash their hands with soap and clean the perineal/genital area with an alcohol pad. Each adolescent was asked to void into a sterile, 100-mL red-topped container (Nipro). Urine samples collected at the two Chichigalpa schools were immediately placed in a cooler for transport to the Chichigalpa Health Center, where urine samples were aliquoted and stored at -20 °C until transported to the National Laboratory Center for Diagnostic and References (CNDR) in Managua for storage at -80 °C. In Masaya and Jinotega, we transported the samples directly to CNDR on the day of collection.

Figure 1. Adolescent study population flow chart



2.3.2 Dipstick analyses in urine

Urine dipstick analyses were conducted on all 245 students who provided urine samples. A urine dipstick (Combur 10UX®, Roche Diagnostics) was immersed in the red-capped urine container and placed into the urine strip reader (Urisys 1100, Roche Diagnostics). The results of the dipstick measures (specific gravity, pH, leukocyte esterase, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, and blood) were recorded on the data collection sheet.

2.3.3 Analysis of biomarkers of kidney damage in urine

As outlined in the study protocol, we selected 200 of the 245 urine samples for analysis of biomarkers of kidney damage. The samples were selected so that there would be 50 per school and 50 each of older males, older females, younger males, and younger females, evenly distributed between the four schools. Samples were randomly selected from within each sex-age category at each school when there was more than the allotted amount of students in that category.

Urine samples were shipped to the Division of Nephrology and Hypertension at the Cincinnati Children's Hospital Medical Center (Cincinnati, OH, USA). Five constituents were analyzed:

1. *Creatinine*, for the purpose of normalizing other urinary biomarkers to account for urine dilution;
2. *Albumin- creatinine ratio (ACR)*, primarily a measure of damage to the glomerulus of the kidney;
3. *Neutrophil gelatinase-associated lipocalin (NGAL)*, primarily a measure of damage to the proximal tubules of the kidney;
4. *N-acetyl--D-glucosaminidase (NAG)*, primarily a measure of damage to the distal tubules; and
5. *Interleukin-18 (IL18)*, primarily a measure of damage to the proximal tubules.

Albumin and creatinine were measured by immunoturbidimetry and a colorimetric modification of the Jaffe reaction, respectively, on a Siemens Dimension Xpand plus HM chemistry analyzer (Siemens Healthcare Diagnostics, Deerfield, IL). Creatinine was reported in milligrams per milliliter of urine (mg/ml). ACR was reported in milligrams per liter of urine (mg/L) but for analysis was converted to micrograms of ACR per milligram of creatinine (μg/mg).

NAG activity was measured using a colorimetric assay (Roche Diagnostics, USA). Briefly, 5 μl of sample was incubated with 100 μl of substrate solution (3-cresolsulfonphthaleinyl-N-acetyl-b-D-glucosaminide) for 20 min at 37 °C. The reaction was stopped with a stop solution containing sodium carbonate and the optical density was measured at 580 nm. The optical density values were subtracted from blank and NAG activity was calculated and reported in milliunits per milliliter of urine (mU/ml), but for analysis was converted to milliunits per milligram of creatinine (mU/mg).

NGAL (Bioporto, Gentofte, Denmark) and IL18 (MBL, Intl., Woburn, MA) were measured by enzyme-linked immunosorbent assay (ELISA) according to the respective manufacturer's instructions. NGAL was reported in nanograms per milliliter of urine (ng/ml) but for analysis was converted to nanograms per milligram of creatinine (ng/mg). IL18 was reported in

picograms per milliliter of urine (pg/ml) but for analysis was converted to picograms per milligram of creatinine (pg/mg).

2.4 Questionnaires

As explained above, at the beginning of the week, the forms for parent consent and the self-administered questionnaire for parents were provided to each student after a short explanation. Each family willing to participate returned both the consent document and the questionnaire with information regarding parents' vital status, education, length of residence in the area, current and longest occupation, whether either parent had ever held a job in the sugar cane industry, parents' medical history (CKD, kidney stones, hypertension and diabetes), and brief questions about the invited child's kidney health and medical history.

Adolescents also filled an assent document and responded to a questionnaire filling out a job history, recent physical activities, length of time walked to school in the morning, current health problems, and frequency of urinary symptoms, if any.

Information from both questionnaires was merged, and a variable called "reported kidney problems" was constructed if either parent reported their child to have been hospitalized because of kidney problems, or if the child specifically reported suffering from kidney health problems.

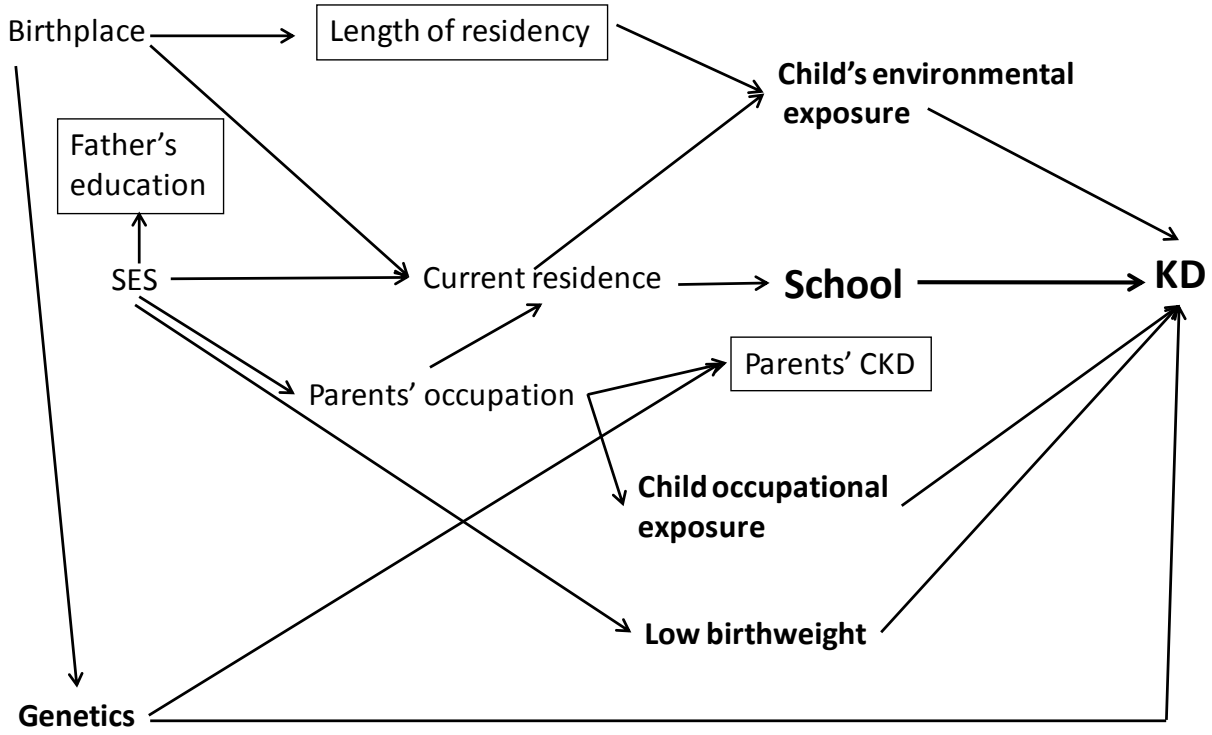
2.5 Data analysis

Biomarker data were evaluated using SPSS statistical software (version 18). The distribution of each biomarker was characterized using graphical displays and summary statistics. When the reported value was less than the limit of detection (LOD), the LOD divided by the square root of 2 was substituted, except for NAG, for which we had no LOD available, and therefore we used a value of 0.02 mU/mg creatinine, which was below the minimum reported value of all the samples. All biomarkers exhibited a lognormal distribution and were therefore natural log-transformed prior to analysis to satisfy normality assumptions.

Linear regression models were used to assess potential predictors of biomarkers of kidney damage. Because the biomarker concentrations were natural log-transformed for the purpose of analysis, the exponentiated β estimates (e^{β}) can be interpreted as the ratio of the mean for each index variable category compared to the mean of the reference variable category (which we refer to as the "mean ratio"). Sex, age, and school were used as the main variables of interest. The students' school is actually a proxy for the child's area of residence, which itself is also a proxy for factors possibly associated with both residence and kidney damage, i.e., occupational and environmental exposures, infectious diseases, low birth weight, and genetic susceptibility (see Figure 2).

Parents' history of CKD, fathers' education (as a proxy for socio-economic status, SES), and length of residence (as a proxy for exposure to community environmental or parents' occupational exposures) were entered in the models to adjust for confounding based on the causal model in Figure 2 and their effect on the estimate of association (>10% change) between school and the kidney damage biomarkers. Although sex and age were not confounders because they were balanced across schools as part of the study design, which we confirmed empirically, we also included sex and age (as a continuous variable) as covariates in all models because it increased the precision of the estimate for some biomarkers. We report analyses adjusted only for sex and age and adjusted for the total set of covariates so that readers may have both available for comparison.

Figure 2. Causal pathway underlying assumptions in the study mathematical modeling*



*In bold, underlying causal hypothesis

2.6 Comparison with results from other studies

Of the four biomarkers measured in this study, NGAL, IL-18, and NAG have been used in many clinical settings by several authors for diagnosis and prognostication of acute kidney injury (AKI). Due to the unavailability of a histological gold standard in these studies, identifying cutoffs for clinical kidney injury has been challenging, and there currently are no accepted cut points for what constitutes an elevated level. Thus, interpretation of the results of biomarkers among Nicaraguan adolescents was approached by comparing the distributions in this population with other published cohorts. A review of studies (mostly published in 2003 through 2012 as

well as unpublished data) was performed to ascertain the reported levels of these biomarkers in different clinical settings such as healthy individuals as normal controls, hospitalized with AKI, critically ill, sepsis, cardiac surgery, post transplant injury, contrast induced nephropathy (a condition occurring after a patient receives an iodinated radiocontrast agent used for an imaging procedure), hepatorenal syndrome (HRS; progressive kidney failure in a person with cirrhosis of the liver), HIV, autosomal dominant polycystic kidney disease (ADPKD), and CKD.

We included 12 published studies and data from two unpublished studies (data requested from investigators). If there was more than one study in the same clinical setting, we included the study with the largest sample size. We created forest plots to compare the range of biomarkers in Nicaraguan adolescents with those reported for normal individuals as well as several other clinical settings. The values of biomarkers were log transformed to create forest plots. The reported values are median with interquartile ranges unless mentioned otherwise. Values both corrected and uncorrected for creatinine were compared for IL-18 and uNGAL. However, for NAG we only compared the values that were corrected for urinary creatinine, as most authors published only creatinine-corrected values for NAG.

The characteristics of the 14 studies are described in Table 1. The median sample size was 163 (range 29 – 1219), with a total of 4,404 participants. Three studies provided data for NAG, 8 studies for NGAL and 9 studies for IL-18. The average age of participants in these studies ranged from 51.6 to 71 years. Although the average age in these studies is older than the adolescent population of this study, no studies have been published in an adolescent population. The only other studies on children have been among infants, and the biomarker levels for adolescents in this study should be more similar to those for healthy adults in the age range of the studies referenced here than levels among infants.

Table 1. Characteristics of studies included in comparative analysis of urinary biomarkers.

Reference	Sample Size (N)	Age (y)	Clinical Setting	Biomarkers	Biomarker Measurement Time	Assay / Lab
Siew, et al. 2009	451	53.5	CI	NGAL	Enrollment*	ELISA kit (Antibody Shop, Gentofte, Denmark)
Bagshaw, et al. 2010	83	63.5	CI / Sepsis	NGAL	Enrollment*, 12h, 24h	ARCHITECT assay (Abbott)
Siew, et al. 2010	588	53.5	CI	IL-18	Enrollment*	Human IL-18 ELISA Kit MBL International, Denmark
Lonnemann, et al. 2003	29	-	CKD	IL-18	-	-
Malyszko, et al. 2009	140	63.9	CIN	IL-18	Preprocedure, Post 2h, 4h, 8h, 24h*, 48h	R&D Kit, Abington, UK
Parikh, et al. 2011	1219	71	CS	IL-18, NGAL	Preop, Postop day 1 q6h* then daily for 5 days	ARCHITECT assay (Abbott)
Hall, et al. 2011	249	66.2	Hosp / AKI	IL-18, NGAL	Enrollment*	ELISA Kit (Antibody Shop, Denmark)
Hall, et al. 2010	91	51.6	Tp	IL-18, NGAL	Q6h postop starting at 0h* x 4 then daily x 2d	ELISA NGAL (AbS, Denmark), Human IL-18 ELISA Kit (MBL, Japan)
Parikh, et al. 2012	107	32.1	ADPKD	IL-18, NGAL	Enrollment* then annually x 3	ELISA NGAL (AbS, Denmark), Human IL-18 ELISA Kit (MBL, Japan)
Belcher, et al.†	187	55.1	HRS	IL-18, NGAL		
WIHS†	895	-	HIV	IL-18, NGAL		
Han, et al. 2008	30	40.8	ER	NAG	Enrollment*	Calorimetric Assay (Roche App. Sciences, IN)
Han, et al. 2009	90	64.3	CS	NAG	PreOp, Immediate PostOp*, 3h, 18h, 24 h	Calorimetric Assay (Roche App. Sciences, IN)
Liangos, et al. 2007	201	65	Hosp / AKI	NAG	Enrollment*	Colorimetric Assay (Boehringer Mannheim, Mannheim, Germany)

***Values at this time point were used in comparative analysis.**
†Note: Belcher and WIHS are unpublished studies.
CI, Critically ill; CIN, Contrast induced nephropathy; CS, Cardiac surgery**; Hosp, Hospitalized; Tp, Transplant**

III. RESULTS & DISCUSSION

3.1 Study population characteristics

A total of 245 students were enrolled and provided questionnaire information. Table 2 summarizes the data on the parents. Parents had resided at their current address for a median time of 13 years (range 1 week-57 years) and in the same Department within Nicaragua for a median of 33 years (range 2-57). The median length of residence at their current address was approximately 5-6 years less for parents of students at School 4 (in southern Chichigalpa) than for parents of students at any of the other schools. However, there were only small differences in the length of residence in the same department. Almost no parents of students at School 4 had completed a university or professional education compared to a range of 17%-30% at the other schools. Schools 2 (Masaya) and 4 showed the highest percentage of fathers (51 and 54% respectively) who did not have education beyond primary school, compared to about a third of those at the other schools. Among mothers, the percentage with no more than a primary school education was much higher at School 4 (62%) than among the other schools (28%-42%). As expected based on the study design, virtually all students (94%) at School 4 had at least one parent who currently or in the past worked in the sugar cane industry, while the parent of only one student in the other three schools combined had worked in sugar cane.

At all schools, the mothers of virtually all students were still living. At School 4 in southern Chichigalpa, 13% of students' fathers had died compared to 5%-6% at the other schools. The same pattern was observed for self-reported CKD, with relatively low rates (2-6%) among mothers across all schools, while 36% of fathers at School 4 in southern Chichigalpa reported CKD compared to 2%-6% at the other schools.

The data for students is shown in Table 3. The sex distribution among students was quite similar across schools, while the proportion of younger students was slightly higher at School 1 (Jinotega) and 4. We also assessed the type of transport and time walked to get to the school, since it is possible that students who had walked for a substantial period of time might have had a transient increase in biomarker levels. Five students reported taking public (bus) or private (car, motorbike) transportation to commute to school. Among those who walked, the median time to get to school was 10 minutes, with a minimum of 1 and a maximum of 60 minutes. Only 5% of children at Schools 1 and 4 walked ≥30 minutes compared to 13%-18% at Schools 2 and 3.

Table 2. Characteristics of parent population

Schools	1. Jinotega	2. Masaya	3. North Chichigalpa *	4. South Chichigalpa	TOTAL
	n=63	n=52	n=63	n=67	n=245
	n (%)	n (%)	n (%)	n (%)	n (%)
Father education					
No school	1 (1.5%)	2 (5%)	4 (7%)	6 (10%)	13 (6%)
Primary	15 (24.5%)	19 (46%)	15 (27%)	28 (44%)	77 (34.5%)
Secondary	31 (51%)	14 (33%)	23 (41%)	27 (43%)	95 (43%)
University/professional	14 (23%)	7 (17%)	14 (25%)	2 (3%)	37 (16.5%)
Mother education					
No school	0 (0%)	1 (2%)	0 (0%)	3 (5%)	4 (2%)
Primary	17(28%)	17 (40%)	20 (34%)	34 (57%)	88 (39%)
Secondary	26 (43%)	16 (37%)	28 (47%)	23 (38%)	93 (42%)
University/professional	18 (29%)	9 (21%)	11 (19%)	0 (0%)	38 (17%)
Years lived in the same address					
<5	8 (13%)	5 (10%)	1 (2%)	3 (4%)	17 (7%)
5-9	6 (9.5%)	8 (16%)	10 (16%)	5 (8%)	29 (12%)
10-19	23 (36.5%)	18 (35%)	28 (44%)	51 (77%)	120 (49%)
20+	26 (41%)	20 (39%)	24 (38%)	7 (11%)	77 (32%)
Years lived in the same region (department)					
<5	0 (0%)	1 (2%)	0 (0%)	0 (0%)	1 (0.4%)
5-9	0 (0%)	0 (0%)	2 (3%)	0 (0%)	2 (0.8%)
10-19	10 (16%)	13 (27%)	19 (31%)	17 (26%)	59 (24.7%)
20+	53 (84%)	35 (71%)	40 (66%)	49 (74%)	177 (74.1%)
Sugar cane parent job					
Father or mother worked in sugar cane	0 (0%)	0 (0%)	1 (2%)	63 (94%)	64 (26%)
Parents' health reported background					
Either parent reported CKD	7 (11%)	3 (6%)	4 (6%)	26 (39%)	40 (16%)
Father					
CKD	4 (6%)	1 (2%)	4 (6%)	24 (36%)	33 (16%)
Kidney stones	7 (11%)	1 (2%)	1 (2%)	6 (9%)	15 (7%)
Hypertension	8 (13%)	6 (12%)	6 (10%)	12 (18%)	32 (13%)
Diabetes	7 (11%)	3 (6%)	5 (8%)	3 (5%)	18 (9%)
Deceased	3 (5%)	3 (6%)	3 (5%)	9 (13%)	18 (8%)
Mother					
CKD	4 (6%)	2 (4%)	1 (2%)	3 (5%)	10 (5%)
Kidney stones	6 (10%)	4 (8%)	1 (2%)	3 (5%)	14 (7%)
Hypertension	11 (18%)	11 (21%)	8 (13%)	12 (18%)	42 (20%)
Diabetes	2 (3%)	2 (4%)	4 (6%)	4 (6%)	12 (6%)
Deceased	0 (0%)	0 (0%)	3 (5%)	2 (3%)	5 (2%)

*4 adolescents lived in Posoltega.

Table 3. Characteristics of child population

Schools	1. Jinotega	2. Masaya	3. North Chichigalpa	4. South Chichigalpa	TOTAL
	n=63	n=52	n=63	n=67	N=245
	n (%)	n (%)	n (%)	n (%)	n (%)
Sex					
Male	33 (52%)	25 (48%)	32 (51%)	31 (46%)	121 (49%)
Female	30 (48%)	27 (52%)	31 (49%)	36 (54%)	124 (51%)
Age group					
12-15y	37 (59%)	25 (48%)	33 (52%)	38 (57%)	133 (54%)
16-18y	26 (41%)	27 (52%)	30 (48%)	29 (43%)	112 (46%)
Minutes walked to school					
<10	15 (26%)	7 (16%)	8 (15%)	37 (62%)	67 (31%)
10-29	39 (69%)	30 (67%)	38 (72%)	20 (33%)	127 (59%)
≥30	3 (5%)	8 (18%)	7 (13%)	3 (5%)	21 (10%)

As stated previously, 200 of the 245 students included in the study also had samples analyzed for the biomarkers NGAL, NAG, IL-18, and ACR. The 200 students were selected to provide a balanced distribution for school, sex, and age category. Other characteristics had a distribution similar to that observed among all 245 participants.

3.1 Dipstick data

Dipstick data were available on all 245 study participants. Urine dipsticks were used to semi-quantitatively assess urine specific gravity, pH, leukocyte esterase, nitrite, protein, glucose, ketones, urobilinogen, bilirubin, and blood (Table 4).

Table 4. Summary of dipstick data for all study participants

	Schools (city)			
	1. Jinotega	2. Masaya	3. North Chichigalpa	4. South Chichigalpa
N=245	n=63	n=52	n=63	n=67
Urine dipstick measures	n (%)	n (%)	n (%)	n (%)
SPECIFIC GRAVITY				
1,005	4 (6%)	2 (4%)	7 (11%)	16 (24%)
1,010	14 (22%)	19 (37%)	18 (29%)	25 (37%)
1,015	18 (29%)	12 (23%)	20 (32%)	20 (30%)
1,020	19 (30%)	12 (23%)	14 (22%)	6 (9%)
1,025	4 (6%)	3 (6%)	3 (5%)	0 (0%)
1,030	4 (6%)	4 (8%)	1 (2%)	0 (0%)
pH				
5	41 (65%)	25 (48%)	26 (41%)	19 (28%)
6	10 (16%)	11 (21%)	19 (30%)	24 (36%)
6.5	1 (2%)	3 (6%)	3 (5%)	7 (10%)
7	8 (13%)	9 (17%)	10 (16%)	12 (18%)
8	3 (5%)	4 (8%)	5 (8%)	5 (8%)
LEUKOCYTE ESTERASE				
Neg	57 (90%)	46 (88%)	58 (92%)	61 (91%)
+1	3 (5%)	2 (4%)	0 (0%)	1 (1.5%)
+2	1 (2%)	4 (8%)	2 (3%)	1 (1.5%)
+3	2 (3%)	0 (0%)	3 (5%)	4 (6%)
NITRITE				
Neg	63 (100%)	51 (98%)	61 (97%)	65 (97%)
Pos	0 (0%)	1 (2%)	2 (3%)	2 (3%)
BLOOD				
Neg	55 (87%)	45 (86%)	57 (90%)	60 (90%)
+1	2 (3%)	4 (8%)	1 (2%)	3 (4%)
+2	1 (2%)	3 (6%)	2 (3%)	2 (3%)
+3	2 (3%)	0 (0%)	2 (3%)	0 (0%)
+4	3 (5%)	0 (0%)	1 (2%)	2 (3%)
PROTEIN				
Neg	63 (100%)	48 (92%)	61 (97%)	66 (99%)
+1	0 (0%)	3 (6%)	2 (3%)	0 (0%)
+2	0 (0%)	0 (0%)	0 (0%)	0 (0%)
+3	0 (0%)	1 (2%)	0 (0%)	1 (1%)
GLUCOSE				
Neg	63 (100%)	50 (96%)	63 (100%)	67 (100%)
+1	0 (0%)	1 (2%)	0 (0%)	0 (0%)
+2	0 (0%)	1 (2%)	0 (0%)	0 (0%)
KETONES				
Neg	63 (100%)	52 (100%)	62 (98%)	67 (100%)
+1	0 (0%)	0 (0%)	1 (2%)	0 (0%)
UROBILINOGEN				
Neg	63 (100%)	52 (100%)	60 (95%)	67 (100%)
+1	0 (0%)	0 (0%)	2 (5%)	0 (0%)
BILIRUBIN				
Neg	63 (100%)	52 (100%)	63 (100%)	67 (100%)

We will focus our analysis and discussion on protein, glucose, nitrite, leukocyte esterase, and blood. Leukocyte esterase is an enzyme produced by white blood cells, such that a positive test typically indicates the presence of white blood cells in the urine. The presence of white cells in urine can be an indication of infection (e.g., urinary tract infection, UTI) but can also be an indication of inflammation (e.g., crystals/stones, tubular injury). Accordingly, leukocyte esterase tests are often interpreted in combination with other measurements, such as tests for nitrite and hemoglobin in urine, as well as the actual presence of white blood cells in the urine on urine microscopy. The presence of nitrites in urine is a specific but not sensitive test that is most consistent with the presence of gram-negative bacteria, the most common urinary pathogen. Typically, testing positive for leukocyte esterase and/or nitrite is suggestive of a UTI and is usually followed by a confirmatory urine culture. Both leukocyte esterase and nitrites were only very rarely detected in the study population. Nitrites were detected only in 5 adolescents (2%). Only 23 (9.4%) adolescents had a positive leukocyte esterase, of whom 22 were females evenly distributed among schools.

We further analyzed whether leukocyte esterase positivity in females was associated with biomarkers of kidney injury. As shown in Table 5, the mean ratio of NGAL values was higher in leukocyte esterase positive females compared to leukocyte esterase negative females.

Table 5. Analysis of biomarkers* by leukocyte esterase positivity in females

	Mean Ratio	LCL	UCL
ACR (ug/mg Crea)	1.10	0.73	1.66
NGAL (ng/mg Crea)	2.13	1.46	3.12
NAG (mU/mg Crea)	0.75	0.49	1.13
IL-18 (pg/mg Crea)	1.11	0.74	1.66

*Biomarkers were natural log-transformed. LCL: Low confidence interval level. UCL: Upper confidence interval level.

The most common cause of a positive test for blood in urine is the presence of red blood cells (hematuria). Other causes include the presence of myoglobin in the urine (from rhabdomyolysis [muscle damage]) or hemoglobin itself in the urine (from intravascular hemolysis [breakdown of red blood cells]). Injury anywhere along the genitourinary tract can potentially cause hematuria. Causes include bladder lesions, ureteral lesions (e.g., trauma from kidney stones), inflammation in the setting of conditions like urinary tract infections and kidney stones and crystals, and kidney lesions, ranging from benign causes like thin basement membrane disease to severe glomerulonephritis. In our population, 28 (11.4%) adolescents tested positive for hemoglobin, with 22 of those 28 being females evenly distributed among schools. Only IL-18 showed a higher relative mean when we analyzed whether blood positivity was associated with higher values of biomarkers of kidney injury in females (Table 6). Although self-reported current menstruation was an exclusion criterion, it is possible that some of the positive blood results could be due to that fact.

Table 6. Analysis of biomarkers* by blood positivity in females

	Mean Ratio	LCL	UCL
ACR (ug/mg Crea)	1.26	0.83	1.91
NGAL (ng/mg Crea)	1.01	0.66	1.53
NAG (mU/mg Crea)	0.91	0.59	1.40
IL-18 (pg/mg Crea)	1.64	1.10	2.44

Biomarkers were Ln-transformed. LCL: Low confidence interval level. UCL: Upper confidence interval level.

One notable result from dipstick data is that protein (7 cases or 2.9%) or glucose (2 cases or 0.8%) was only rarely present in the urine of study participants, suggesting little evidence of glomerular disease, which is consistent with the finding described below for urinary Albumin-Creatinine Ratio (ACR).

In summary, urine characteristics measured by urine dipstick that might suggest kidney damage do not appear to be elevated and do not differ by school. However, it is possible that the presence of leukocyte esterase and/or blood might be associated with kidney damage among those girls with elevated values.

3.2 Urine creatinine

We evaluated urine creatinine concentrations and urine specific gravity to ascertain whether Nicaraguan adolescents were in a chronic pre-renal state (a state of renal hypoperfusion [decreased blood flow] that may result from different hemodynamic misbalances, such as dehydration) and have concentrated urine, which would falsely increase their urinary biomarker concentrations to a level consistent with kidney injury. Table 7 shows the distribution of urine creatinine concentration for the overall population and by sex, age group and school.

Table 7. Distribution of urine creatinine concentration, by sex, age group and school

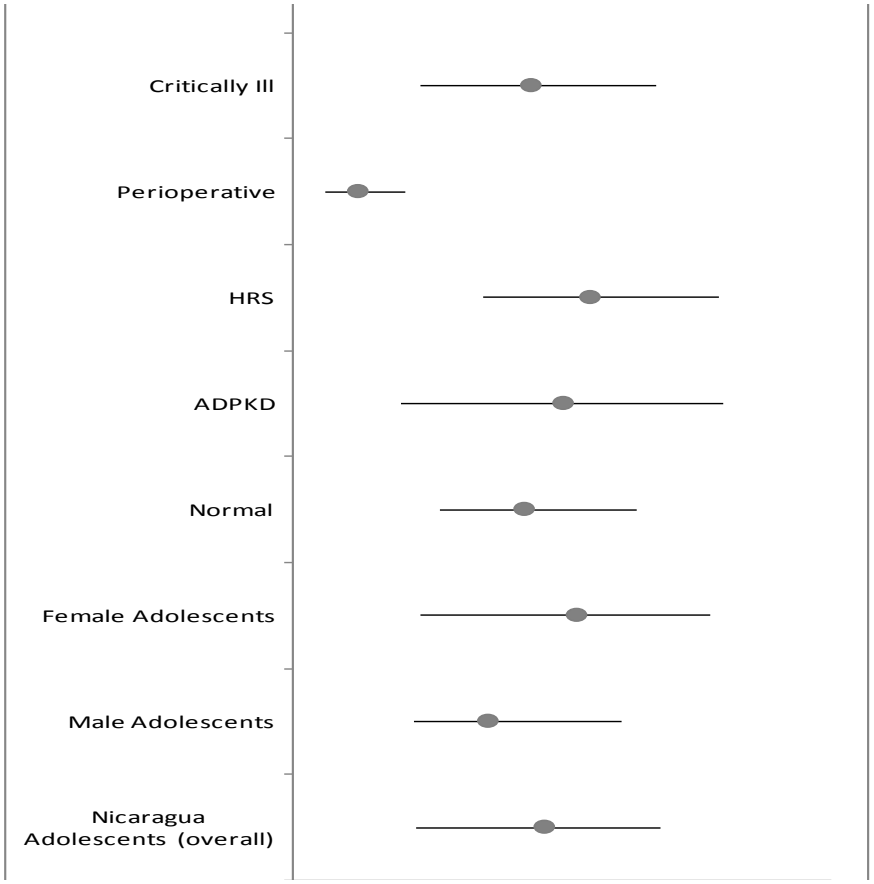
Creat mg/ml	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	1	0.66	0.07	0.46	0.93	1.37	3.55
Sex								
Female	100	1.07	0.65	0.07	0.47	1.05	1.55	3.20
Male	100	0.92	0.66	0.11	0.45	0.72	1.22	3.55
Age group								
12-15	103	0.94	0.64	0.07	0.37	0.83	1.37	2.79
16-18	97	1.05	0.68	0.12	0.54	0.94	1.43	3.55
School								
Jinotega	50	1.26	0.71	0.20	0.71	1.15	1.73	3.55
Masaya	50	0.80	0.58	0.12	0.39	0.63	1.09	3.20
North C.	50	1.22	0.68	0.23	0.64	1.17	1.62	2.76
South C.	50	0.70	0.45	0.07	0.32	0.62	0.97	1.72

We compared the urinary creatinine concentration values in adolescents with those reported for healthy controls as well as individuals with different clinical conditions (Figure 3). The median urinary creatinine concentration in adolescents analyzed in this study was 93 mg/dl, with an interquartile range (IQR), which represents the 25^{th} and 75^{th} percentiles, of 46 mg/dl to 137 mg/dl. These values are comparable to the value of 85.5 mg/dl (IQR 54.5-127.5) for healthy individuals. The median urinary creatinine concentration value in male adolescents was lower as compared to female adolescents, 72 mg/dl (IQR 45-122) vs. 105 mg/dl (IQR 47-155) respectively ($P<0.01$). This finding is unusual since boys usually excrete significantly more creatinine compared with girls before and during puberty (reflecting greater muscle mass) (Skinner et al., 1996). Nevertheless, this finding was corroborated when we also examined specific gravity of the urine in dipstick data (Table 8). The cumulative percent in three highest hexiles of urine specific gravity (1.020, 1.025 and 1.030) in females was 34.7 vs. 22.3 in males ($p=0.0005$), again suggesting that urine concentration was higher in female adolescents (see Table 9). One possible explanation for the difference in urine concentration is that female adolescents may restrict their fluid intake because they feel more uncomfortable than boys urinating in public toilets (such as those in schools) for hygiene and cultural reasons. This phenomenon has been reported anecdotally in Nicaragua and other countries.

Table 8. Urine specific gravity by sex

Specific gravity	Females (n=100)	Males (n=100)
1,005	13 (10.5%)	16 (13.2%)
1,010	30 (24.2%)	46 (38%)
1,015	38 (30.6%)	32 (26.4%)
1,020	29 (23.4%)	22 (18.2%)
1,025	6 (4.8%)	4 (3.3%)
1,030	8 (6.5%)	1 (0.8%)

Figure 3. Comparative analysis of urinary creatinine in Nicaraguan adolescents with urinary creatinine in other clinical settings.



Transplant (Hall et al.) Critically Ill (Siew et al.) Perioperative (Parikh et al.) ADPKD (Parikh et al.)
Note: Data reported for HRS cohort is unpublished. For normal, we assumed cardiac surgery patients' preoperative values will be closest to normal individuals.

Urine Biomarkers of Kidney Injury

3.3.1 Urine ACR

Elevated urine albumin, normalized for creatinine (ACR), is a measure of kidney damage that most often indicates increased glomerular permeability to proteins. ACR calculated from a single urine specimen collected at one point in time has similar utility to measuring albumin in a 24-hour sample. The presence of albumin in the urine can also indicate tubular dysfunction, reflecting an inability of the proximal tubules to fully reabsorb albumin; in this setting, the urine ACR typically is lower than in cases of glomerular proteinuria. Albumin is the most common circulating protein in blood and is not normally present in urine; in settings of glomerular disease (most commonly diabetes or primary glomerular kidney diseases), small amounts of albumin may leak into urine. When the amount of albumin leaking across the glomerular capillaries exceeds the ability of the tubules to reabsorb albumin, albuminuria results. Very low levels (ACR of 30-299 mg/g) are categorized as microalbuminuria, while higher levels (ACR 300+ mg/g) are categorized as macroalbuminuria (National Kidney Foundation, 2002). Macroalbuminuria almost always indicates significant glomerular disease, and is commonly seen in hypertensive and diabetic nephropathies. There was one case of macroalbuminuria among the students in our study. Table 9 shows the distribution of microalbuminuria levels by school.

Table 9. Microalbuminuria results in adolescents by school

ACR	**<30ug/mg n(%)**	**≥30 ug/mg n(%)**
Overall	184 (92%)	16 (8%)
Sex		
Female	92 (92%)	8 (8%)
Male	92 (92%)	8 (8%)
Age group		
12-15	93 (90.3%)	10 (9.7%)
16-18	91 (93.8%)	6 (6.2%)
School		
Jinotega	43 (86%)	7 (14%)
Masaya	46 (92%)	4 (8%)
North Chichigalpa	48 (96%)	2 (4%)
South Chichigalpa	47 (94%)	3 (6%)*

*Only one boy from School 4 at South Chichigalpa presented a value of ACR higher than 300 ug/mg creatinine.

Tables 10-11 show the distribution of urine albumin and ACR (normalized by urine creatinine) for the overall population and by sex, age group and school. Figure 4 shows the overall distribution.

Table 10. Distribution of urine albumin by sex, age group and school

Alb ng/ml	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	16.7	50.5	0.2	2.9	5.3	10.5	564.7
Sex								
Female	100	14.9	30.0	0.2	3.5	7.1	13.8	266.8
Male	100	18.4	65.0	0.2	2.5	4.1	7.3	564.7
Age group								
12-15	103	15.3	38.7	0.2	2.7	4.8	9.7	266.8
16-18	97	18.1	60.8	0.7	3.0	6.0	10.7	564.7
School								
1 (Jinotega)	50	21.4	41.2	0.6	3.8	6.2	26.7	266.8
2 (Masaya)	50	12.0	29.7	0.4	2.5	4.2	8.0	201.2
3 (North Chichigalpa)	50	15.4	37.4	1.2	4.3	6.7	10.4	230.4
4 (South Chichigalpa)	50	17.8	79.5	0.2	2.0	3.8	7.9	564.7

Table 11. Distribution of urine ACR (normalized by creatinine) by sex, age group and school

ACR µg/mg Crea		Mean	sd	Min	P25	P50	P75	Max
Overall	200	17.7	59.5	1.1	4.6	6.2	10.5	753.9
Sex								
Female	100	13.6	22.1	1.2	5.4	7.5	11.5	182.4
Male	100	21.8	81.3	1.1	4.1	5.2	7.7	753.9
Age group								
12-15	103	15.9	32.3	1.8	4.7	5.9	8.6	182.4
16-18	97	19.7	78.9	1.1	4.5	6.5	11.1	753.9
School								
1 (Jinotega)	50	17.5	30.9	1.4	4.0	5.3	17.4	182.4
2 (Masaya)	50	16.3	35.5	1.1	4.0	5.3	8.8	218.5
3 (North Chichigalpa)	50	13.3	30.6	2.8	4.6	6.1	10.3	167.9
4 (South Chichigalpa)	50	23.8	105.8	1.75	4.7	6.0	10.8	753.9

Figure 4. Distribution of urinary ACR (µg/mg creatinine)

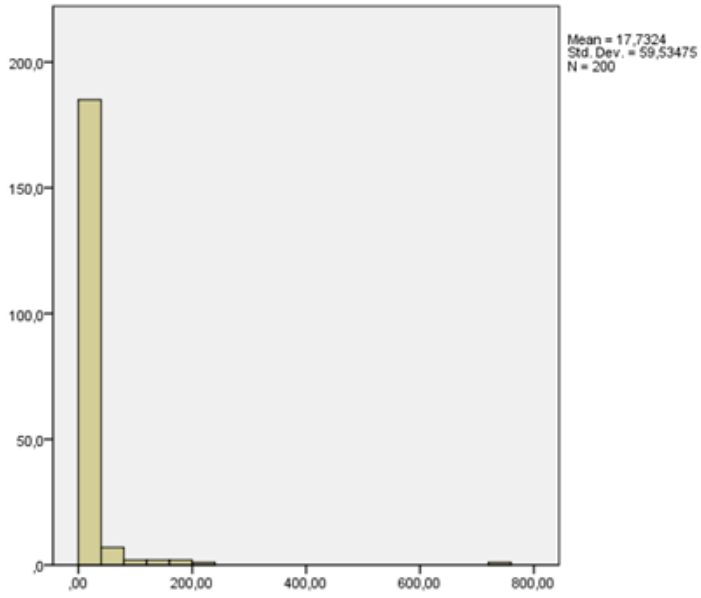


Table 12 presents the results for the linear regression models evaluating the association between ACR concentration and sex, age, and school, adjusted for confounding. Boys had lower ACR concentrations than girls. In addition, girls at schools 2-4 had lower concentrations than girls at school 1.

Table 12. Multivariate analysis of ln(ACR) by sex, age and school.

		ACR			
	N	Mean Conc. ug/mg Crea	Geometric mean ug/mg Crea	Mean Ratio (95% CI)	Mean Ratio (95% CI)
Overall	200	17.7	7.68	N/A	N/A
Sex					
F	100	13.6	8.61	Ref	Ref
M	100	21.8	6.85	0.79 (0.61-1.04)a	0.84 (0.63-1.12)d
Age					
1 year increases	200	NA	NA	0.96 (0.89-1.04)b	0.95 (0.88-1.03)e
School					
1 (Jinotega)	50	17.5	8.16	Ref	Ref
2 (Masaya)	50	16.3	7.79	0.97 (0.66-1.42)c	0.99 (0.65-1.51)f
3 (North Chichigalpa)	50	13.3	7.25	0.89 (0.61-1.30)c	0.90 (0.60-1.35)f
4 (South Chichigalpa)	50	23.8	7.54	0.93 (0.64-1.36)c	0.82 (0.53-1.28)f
Sex:FEMALE					
School					
1 (Jinotega)	25	22.9	11.44	Ref	Ref
2 (Masaya)	25	11.9	7.26	0.64 (0.41-1.02)a	0.72 (0.44-1.17)d
3 (North Chichigalpa)	25	8.6	7.79	0.69 (0.43-1.09)a	0.72 (0.44-1.17)d
4 (South Chichigalpa)	25	11.1	8.48	0.76 (0.48-1.20)a	0.64 (0.37-1.11)d
Sex: MALE					
School					
1 (Jinotega)	25	12.2	5.83	Ref	Ref
2 (Masaya)	25	20.7	8.36	1.47 (0.80-2.69)a	1.52 (0.75-3.07)d
3 (North Chichigalpa)	25	18.0	6.75	1.16 (0.64-2.13)a	1.09 (0.58-2.06)d
4 (South Chichigalpa)	25	36.5	6.70	1.15 (0.63-2.10)a	1.06 (0.53-2.12)d

a. Adjusted for age b. Adjusted for sex c. Adjusted for age and sex d. Adjusted for age, length of residence in the same address, father's education, parents' CKD e. Adjusted for sex, length of residence in the same address, father's education, parents' CKD f. Adjusted for sex, age, length of residence in the same address, father's education, parents' CKD

3.3.2 Urinary NGAL

Neutrophil gelatinase-associated lipocalin (NGAL) is an iron-transporting protein produced in the distal tubules. NGAL expression is substantially upregulated in kidney tubules that are acutely damaged (Devarajan 2008). NGAL is easily detected in both serum and urine within hours of initial insult to the kidney, providing earlier detection of kidney injury than can be achieved by measuring serum creatinine (Devarajan 2008). It has been postulated that NGAL is upregulated in response to injured tubular epithelium and may be responsible for regeneration of epithelium. Thus, its main role may be reparative as opposed to IL-18, which is an injury mediator. NGAL may also identify those patients who will develop CKD (Bolignano et al. 2009; Devarajan 2008).

Tables 13-14 and Figure 5 show the distribution of NGAL for the overall population and by sex, age group and school.

Table 13. Distribution of urine NGAL by sex, age group and school

NGAL ng/ml	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	24.8	42.3	0.97	4.2	9.9	26.0	390.5
Sex								
Female	100	42.5	54.1	1.6	11.2	25.1	58.5	390.5
Male	100	7.1	5.9	0.97	2.9	5.0	9.2	33.3
Age group								
12-15	103	19.2	32.2	0.97	3.1	0.1	18.1	210.5
16-18	97	30.7	50.4	1.26	6.0	14.6	33.2	390.5
School								
1 (Jinotega)	50	27.2	35.5	1.1	7.4	12.2	35.0	203.3
2 (Masaya)	50	13.9	16.5	1.3	2.8	8.6	18.1	87.6
3 (North Chichigalpa)	50	38.2	68.5	1.5	4.3	10.2	40.5	390.5
4 (South Chichigalpa)	50	19.9	26.6	0.97	3.7	10.0	23.8	116.0

Table 14. Distribution of urine NGAL (normalized by creatinine) by sex, age group and school

NGAL ng/mg Crea	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	24.8	33.8	1.8	6.3	13.3	28.1	307.3
Sex								
Female	100	40.0	41.8	5.8	15.6	25.7	49.6	307.3
Male	100	9.6	8.9	1.8	4.2	7.0	10.9	56.7
Age group								
12-15	103	21	26.4	1.8	5.7	10.6	22.3	122.3
16-18	97	28.9	39.8	2.4	7.9	15.6	33.5	307.3
School								
1 (Jinotega)	50	20.9	21.2	2.5	6.8	12.0	26.1	96.5
2 (Masaya)	50	18.1	19.9	2.5	6.2	13.1	21.9	122.3
3 (North Chichigalpa)	50	31.1	49.7	1.8	5.2	11.4	38.7	307.3
4 (South Chichigalpa)	50	29.1	34.5	2.3	8.2	15.1	34.2	187.9

Figure 5. Distribution of urinary NGAL (ng/mg creatinine)

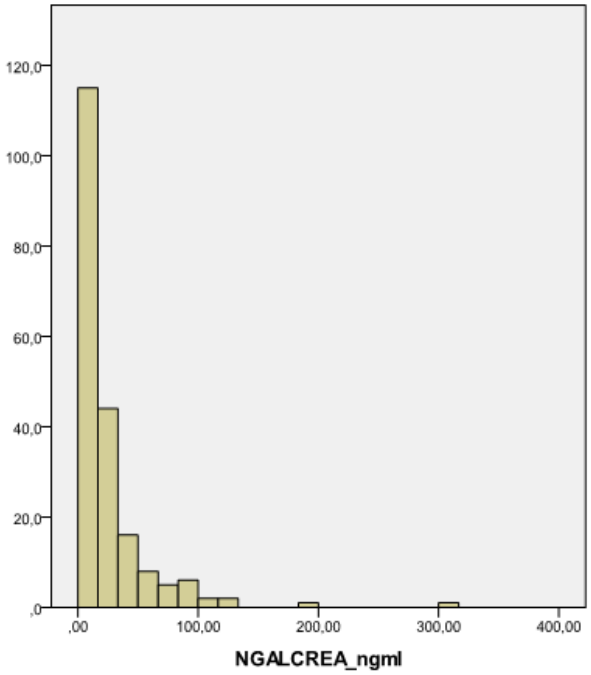


Table 15 presents the results for the linear regression models evaluating the association between NGAL concentration and sex, age, and school, adjusted for confounding. Mean concentrations in boys were 4 times lower than in girls. Each year increased the mean concentration of NGAL by 9%. An 18-year-old subject would have a NGAL concentration that was 51% higher than that of a 13 year old. The mean values of NGAL were 40% higher in School 4 (south Chichigalpa) than in School 1 (Jinotega, the reference school), with both males and females elevated. Female students at School 3 (north Chichigalpa) also had somewhat elevated NGAL levels relative to School 1, though there was no evidence of any elevation among male students.

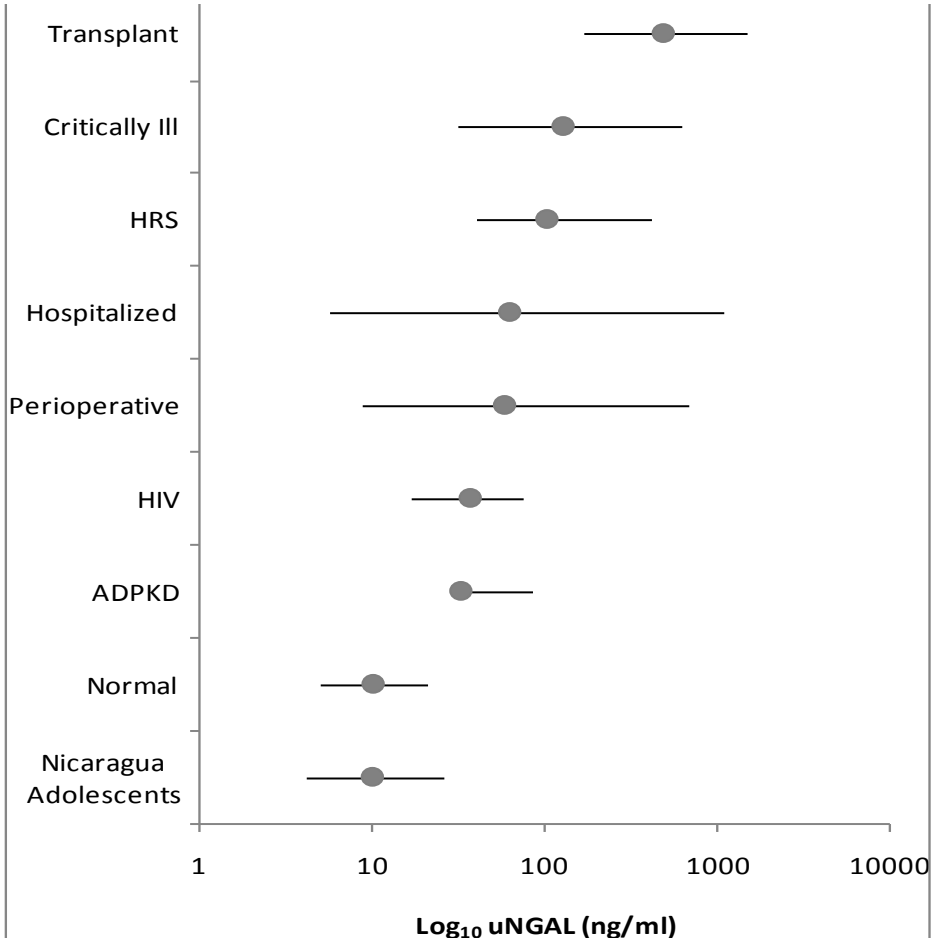
Table 15. Multivariate analysis of ln(NGAL) by sex, age and school.

		NGAL			
	N	Mean Conc. ng/mg Crea	Geometric mean ng/mg Crea	Mean Ratio (95% CI)	Mean Ratio (95% CI)
Overall	200	24.8	14.18	N/A	N/A
Sex					
F	100	40.0	28.04	Ref	Ref
M	100	9.6	7.17	0.26 (0.21-0.32)a	0.25 (0.20-0.32)d
Age					
1 year increases	200	NA	NA	1.09 (1.03-1.16)b	1.09 (1.02-1.16)e
School					
1 (Jinotega)	50	20.9	13.98	Ref	Ref
2 (Masaya)	50	18.1	12.27	0.84 (0.62-1.14)c	0.96 (0.69-1.34)f
3 (North Chichigalpa)	50	31.1	13.66	0.96 (0.71-1.3)c	0.96 (0.70-1.32)f
4 (South Chichigalpa)	50	29.1	17.24	1.20 (0.89-1.63)c	1.40 (0.99-1.97)f
Sex:FEMALE					
School					
1 (Jinotega)	25	33.7	27.70	Ref	Ref
2 (Masaya)	25	24.3	18.49	0.65 (0.42-1.01)a	0.76 (0.45-1.27)d
3 (North Chichigalpa)	25	55.2	35.19	1.25 (0.80-1.94)a	1.28 (0.77-2.13)d
4 (South Chichigalpa)	25	46.9	34.31	1.19 (0.76-1.86)a	1.40 (0.79-2.50)d
Sex: MALE					
School					
1 (Jinotega)	25	8.2	7.06	Ref	Ref
2 (Masaya)	25	11.9	8.14	1.09 (0.74-1.62)a	1.36 (0.87-2.13)d
3 (North Chichigalpa)	25	7.0	5.30	0.75 (0.51-1.10)a	0.77(0.52-1.16)d
4 (South Chichigalpa)	25	11.3	8.67	1.23 (0.84-1.82)a	1.53 (0.98-2.38)d

a. Adjusted for age b. Adjusted for sex c. Adjusted for age and sex d. Adjusted for age, length of residence in the same address, father's education, parents' CKD e. Adjusted for sex, length of residence in the same address, father's education, parents' CKD f. Adjusted for sex, age, length of residence in the same address, father's education, parents' CKD

The median NGAL value for adolescents in this study was similar to values reported for normal individuals (9.9, IQR 4.2-26 vs. 10, IQR 5.1-21.4). The medians reported for urinary NGAL in many clinical settings including but not limited to perioperative setting (46.8, IQR 18.4-107), critically ill (126.6, IQR 32.1-623.1), and transplant patients (483, IQR 169-1498) are significantly higher (Figure 6). A similar pattern was observed when uNGAL values corrected for urinary creatinine were compared (data not shown).

Figure 6. Analysis of the median and range of urine NGAL levels in Nicaragua adolescents compared to urine NGAL in other populations



Transplant (Hall et al.) Critically Ill (Siew et al.) Hospitalized (Hall et al).
Perioperative (Parikh et al.) ADPKD (Parikh et al.)
Note: Data reported for HIV and HRS cohort is unpublished. For normal, we assumed cardiac surgery patients' preoperative values will be closest to normal individuals.

3.3.3 Urinary NAG

N-acetyl-beta-D-glucosaminidase (NAG) is a sensitive biomarker for kidney injury. NAG is an enzyme located in the lysosome, where it plays a role in the breakdown of glycoprotein. A small amount of NAG is normally present in urine, but urinary NAG activity is increased when renal proximal tubular epithelial cells are damaged (Lin et al. 2007). NAG has been used previously as a marker of nephrotoxicity due to exposure to heavy metals, drugs such as certain antibiotics, contrast-induced nephropathy, and also tubule damage due to urolithiasis or urinary tract infections both in adults and children (Price, 1992; Waring, 2001; Devarajan, 2011).

Tables 16-17 and Figure 7 show the distribution of NAG for the overall study population and by sex, age group and school.

Table 16. Distribution of urine NAG by sex, age group and school

NAG mU/ml	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	1.6	1.3	0	0.6	1.2	2.4	7.4
Sex								
Female	100	1.7	1.3	0	0.8	1.5	2.6	7.4
Male	100	1.4	1.2	0	0.6	0.9	2.3	4.7
Age group								
12-15	103	1.7	1.4	0	0.6	1.3	2.7	7.4
16-18	97	1.5	1.1	0	0.7	1.1	2.2	4.7
School								
1 (Jinotega)	50	1.6	1.2	0	0.6	1.2	2.6	4.1
2 (Masaya)	50	1.0	0.9	0	0.5	0.7	1.5	4.0
3 (North Chichigalpa)	50	2.1	1.3	0.3	1.1	2.0	2.8	7.4
4 (South Chichigalpa)	50	1.7	1.4	0.1	0.7	1.2	2.4	6.2

Table 17. Distribution of urine NAG (normalized by creatinine) by sex, age group and school

NAG mU/mg Crea	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	1.8	1.5	0.02	1.04	1.5	2.2	12.9
Sex								
Female	100	1.9	1.4	0.02	1.2	1.6	2.2	9.7
Male	100	1.8	1.7	0.02	1.0	1.4	2.1	12.9
Age group								
12-15	103	2.0	1.4	0.02	1.3	1.7	2.3	9.7
16-18	97	1.7	1.7	0.02	0.8	1.4	2.0	12.9
School								
1 (Jinotega)	50	1.7	2.2	0.02	0.5	1.3	2.2	12.9
2 (Masaya)	50	1.2	0.6	0.02	0.9	1.3	1.6	2.9
3 (North Chichigalpa)	50	1.9	1.0	0.5	1.3	1.6	2.1	5.1
4 (South Chichigalpa)	50	2.5	1.6	0.3	1.4	2.2	3.1	9.7

Figure 7. Distribution of urinary NAG (mU/mg creatinine)

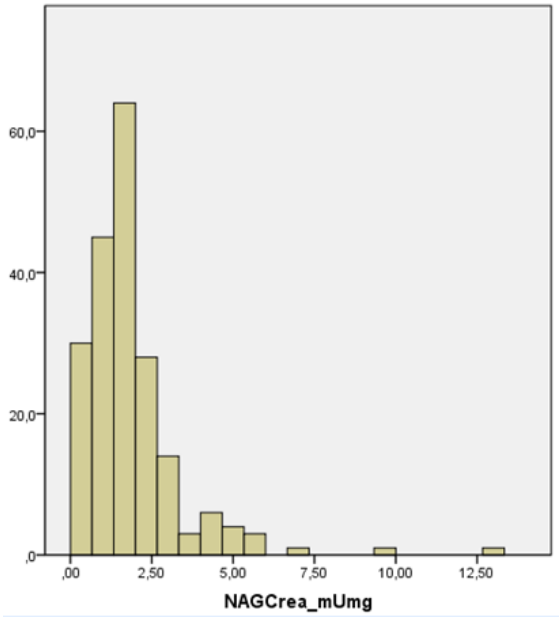


Table 18 presents the results for the linear regression models evaluating the association between NAG concentration and sex, age, and school, adjusted for confounding. Mean concentrations of NAG for boys were 22% lower than for girls, and decreased 8% for each year of age. An 18-year-old subject would have a NAG concentration that was 35% lower than that of a 13-year-old. Taking School 1 (Jinotega) as the reference school, the mean ratio concentrations of NAG were elevated among students at all the remaining schools, particularly at the two schools in Chichigalpa. School 3 (North Chichigalpa) had mean concentrations 2 times higher and School 4 (South Chichigalpa) 2.7 times higher than School 1. Similar results were found when females and males were analyzed separately.

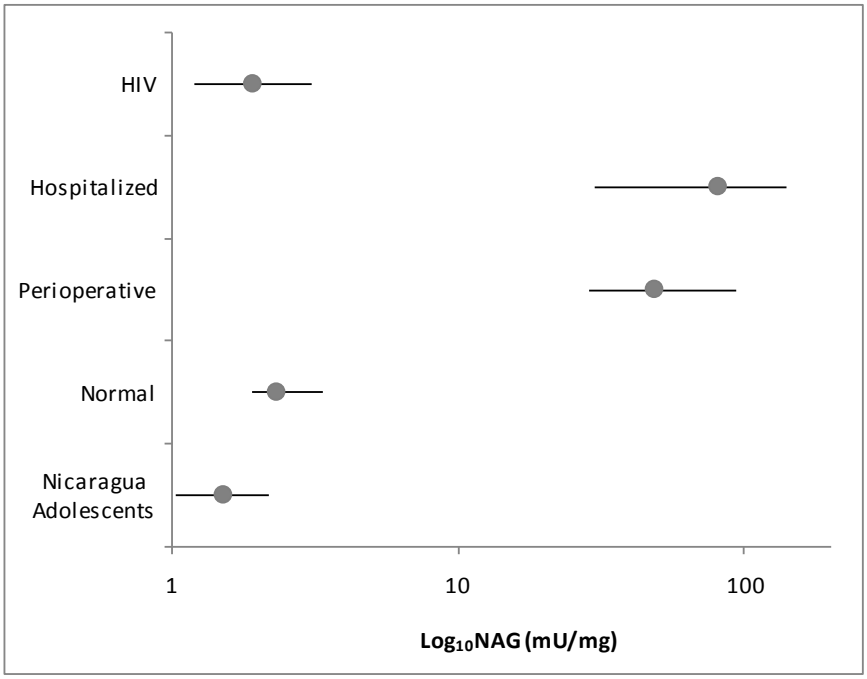
Table 18. Multivariate analysis of ln(NAG) by sex, age and school.

		NAG			
	N	Mean Conc.	Geometric mean	Mean Ratio	Mean Ratio
		mU/mg Crea	mU/mg Crea	(95% CI)	(95% CI)
Overall	200	1.8	1.31	N/A	N/A
Sex					
F	100	1.9	1.47	Ref	Ref
M	100	1.8	1.16	0.79 (0.59-1.05)a	0.78 (0.58-1.05)d
Age					
1 year increases	200	NA	NA	0.90 (0.83-0.97)b	0.92 (0.84-1.00)e
School					
1 (Jinotega)	50	1.7	0.83	Ref	Ref
2 (Masaya)	50	1.2	0.99	1.26 (0.87-1.84)c	1.27 (0.85-1.90)f
3 (North Chichigalpa)	50	1.9	1.66	2.04 (1.40-2.97)c	1.98 (1.35-2.90)f
4 (South Chichigalpa)	50	2.5	2.13	2.64 (1.81-3.85)c	2.69 (1.77-4.10)f
Sex:FEMALE					
School					
1 (Jinotega)	25	1.4	0.89	Ref	Ref
2 (Masaya)	25	1.3	1.19	1.40 (0.92-2.12)a	1.28 (0.86-1.90)d
3 (North Chichigalpa)	25	2.0	1.81	2.09 (1.38-3.17)a	2.00 (1.36-2.94)d
4 (South Chichigalpa)	25	2.9	2.44	2.92 (1.93-4.45)a	2.27 (1.46-3.52)d
Sex: MALE					
School					
1 (Jinotega)	25	2.1	0.77	Ref	Ref
2 (Masaya)	25	1.2	0.83	1.14 (0.60-2.16)a	1.43 (0.70-2.92)d
3 (North Chichigalpa)	25	1.7	1.53	1.99 (1.05-3.77)a	2.15 (1.13-4.07)d
4 (South Chichigalpa)	25	2.2	1.86	2.39 (1.26-4.52)a	3.25 (1.61-6.55)d

a. Adjusted for age b. Adjusted for sex c. Adjusted for age and sex d. Adjusted for age, length of residence in the same address, father's education, parents' CKD e. Adjusted for sex, length of residence in the same address, father's education, parents' CKD
f. Adjusted for sex, age, length of residence in the same address, father's education, parents' CKD

The NAG activity (mU/mg) median value in Nicaraguan adolescents was comparable to values reported for healthy controls (1.5, IQR 1.04-2.2 vs. 2.3, IQR 1.9-3.4) and significantly lower than values reported for hospitalized patients with AKI and cardiac surgery patients (80, IQR 30-140 and 48, IQR 28.5-94, respectively) (Figure 8).

Figure 8. Analysis of the median and range of urine NAG levels (corrected for urine creatinine) in Nicaragua adolescents compared to urine NAG in other populations.



Hospitalized (Liangos, 2007) Perioperative (Han, 2009)
Normal (Han, 2008)
Note: Data reported for HIV cohort is unpublished.

3.3.4 Urinary IL-18

Interleukin-18 (IL-18), a pro-inflammatory cytokine produced during active immune response by macrophages and dendritic cells, is another urinary biomarker that may be elevated early in the course of kidney injury. IL-18 functions by binding to the IL-18 receptor, and together with IL-12 it induces cell-mediated immunity. IL-18 in urine is indicative of renal tubule inflammation, and high concentrations of urinary IL-18 are associated with acute tubule necrosis (Parikh et al. 2005).

Tables 19-20 and Figure 9 show the distribution of IL-18 for the overall population and by sex, age group and school.

Table 19. Distribution of urine IL-18 by sex, age group and school

IL-18 pg/ml	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	115.0	227.1	1.77	20.9	44.9	115.2	1893.7
Sex								
Female	100	190.2	299.8	2.8	45.1	95.9	231.3	1893.7
Male	100	39.8	48.7	1.77	10.6	26.2	41.1	307.6
Age group								
12-15	103	98.1	205.5	2.8	20.5	40.1	96.5	1893.7
16-18	97	133.0	247.8	1.77	21.6	50.2	135.1	1616.5
School								
1 (Jinotega)	50	189.2	354.5	4.2	27.7	65.0	203.6	1893.7
2 (Masaya)	50	86.2	135.1	1.8	7.8	31.5	94.8	599.0
3 (North Chichigalpa)	50	91.8	102.5	5.1	24.8	49.3	129.9	464.8
4 (South Chichigalpa)	50	92.9	218.0	2.8	21.6	33.7	90.9	1528.3

Table 20. Distribution of urine IL-18 (normalized by creatinine) by sex, age group and school

IL-18 pg/mg Crea	n	Mean	sd	Min	P25	P50	P75	Max
Overall	200	100.8	135.4	2.5	28.9	56.7	122.8	976.5
Sex								
Female	100	156.7	166.3	19.6	66.5	117.7	178.8	976.5
Male	100	44.9	53.3	2.5	21.3	32.3	53.1	468.2
Age group								
12-15	103	92.5	115.5	4.9	33.1	52.2	107.2	911.7
16-18	97	109.6	153.7	2.5	26.7	58.6	133.2	976.5
School								
1 (Jinotega)	50	125.8	183.1	9.6	34.5	54.1	165.4	922.1
2 (Masaya)	50	90.3	108.9	2.5	20.4	41.9	119.3	463.8
3 (North Chichigalpa)	50	72.8	59.4	12.7	25.4	48.2	115.3	274.1
4 (South Chichigalpa)	50	114.4	154.1	3.5	38.3	78.8	121.1	976.5

Figure 9. Distribution of urinary IL-18 (pg/mg creatinine)

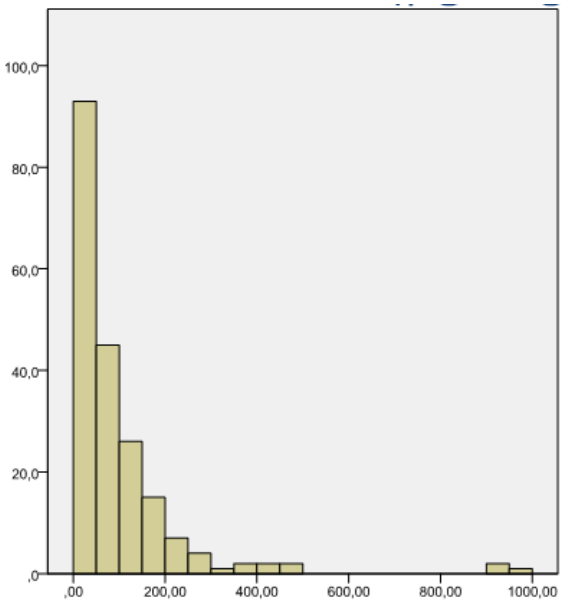


Table 21 presents the results for the linear regression models evaluating the association between IL-18 concentration and sex, age, and school, adjusted for confounding. Mean concentrations of IL-18 were 3.4 times lower in boys than in girls, and there was no association with age. Taking School 1 (Jinotega) as the reference, mean concentrations of IL-18 were lower in School 2 (Masaya) and School 3 (north Chichigalpa), and there was no association with School 4 (south Chichigalpa). However, when analyzed independently, females showed a decreased mean ratio values in all three schools, while males in School 4 showed an increased mean ratio values when compared to School 1.

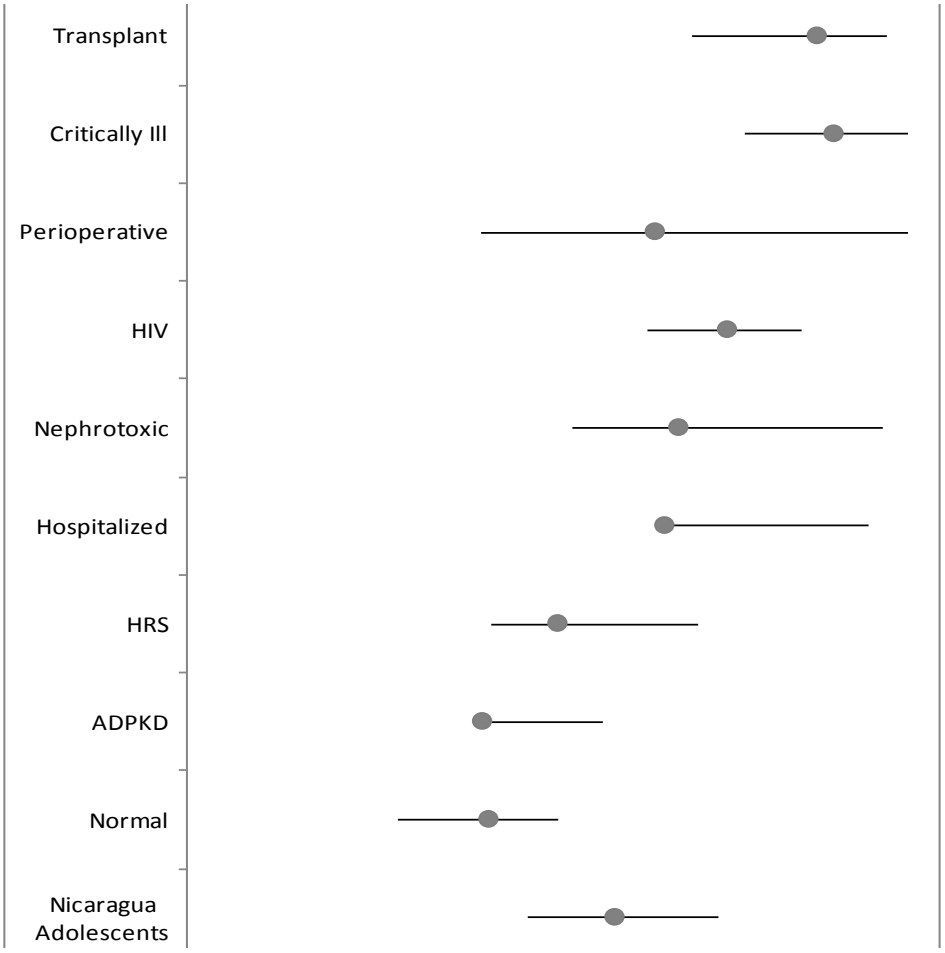
Table 21. Multivariate analysis of ln(IL-18) by sex, age and school.

		IL-18			
	N	Mean Conc. pg/mg Crea	Geometric mean pg/mg Crea	Mean Ratio (95% CI)	Mean Ratio (95% CI)
Overall	200	100.8	58.67	N/A	N/A
Sex					
F	100	156.7	111.06	Ref	Ref
M	100	44.9	31.00	0.28 (0.22-0.35)[a]	0.29 (0.23-0.37)d
Age					
1 year increases	200	NA	NA	0.97 (0.91-1.03)[b]	0.97 (0.91-1.04)e
School					
1 (Jinotega)	50	125.8	70.13	Ref	Ref
2 (Masaya)	50	90.3	44.45	0.64 (0.46-0.89)[c]	0.64 (0.45-0.91)f
3 (North Chichigalpa)	50	72.8	53.47	0.77 (0.55-1.06)[c]	0.75 (0.54-1.05)f
4 (South Chichigalpa)	50	114.4	71.12	1.02 (0.74-1.41)[c]	1.04 (0.72-1.50)f
Sex:FEMALE					
School					
1 (Jinotega)	25	217.1	156.07	Ref	Ref
2 (Masaya)	25	150.8	105.32	0.64 (0.42-0.99)a	0.65 (0.40-1.05)d
3 (North Chichigalpa)	25	112.8	97.61	0.61 (0.39-0.94)a	0.64 (0.40-1.04)d
4 (South Chichigalpa)	25	146.2	94.81	0.57 (0.37-0.88)a	0.57 (0.33-0.98)d
Sex: MALE					
School					
1 (Jinotega)	25	34.5	31.51	Ref	Ref
2 (Masaya)	25	29.7	18.76	0.64 (0.41-0.98)a	0.58 (0.35-0.96)d
3 (North Chichigalpa)	25	32.8	29.29	0.94 (0.61-1.44)a	0.85 (0.54-1.33)d
4 (South Chichigalpa)	25	82.5	53.34	1.68 (1.09-2.59)a	1.62 (0.99-2.66)d

a. Adjusted for age b. Adjusted for sex c. Adjusted for age and sex d. Adjusted for age, length of residence in the same address, father's education, parents' CKD e. Adjusted for sex, length of residence in the same address, father's education, parents' CKD f. Adjusted for sex, age, length of residence in the same address, father's education, parents' CKD

The median value of IL-18 (pg/ml) in Nicaraguan adolescents was higher than that in healthy controls (44.9, IQR 20.9-115.2 vs. 14.6, IQR 6.6-27.6) and was in the range reported for many injury patterns such as hospitalized patients with AKI (70, IQR 0-437) and perioperative injury (135, IQR 50.3-290.3) (Figure 10). A similar pattern was observed when IL-18 values corrected for urinary creatinine were compared (data not shown). A subanalysis of IL-18 values in adolescents from South Chichigalpa school (not shown) where adult CKD prevalence has been reported to be the highest among the 4 schools, did not show any significant difference from adolescents at the other three schools. As noted above, the levels of IL-18 among girls were much higher than among boys. Thus, the overall median value obscures the fact that boys were only modestly elevated (26.2, IQR 10.6-41.1) compared to healthy controls, while girls had levels much more similar to individuals with clinical conditions (95.9, IQR 45.1-231.3).

Figure 10. Analysis of the median and range of urine IL-18 levels in Nicaragua adolescents compared to urine IL-18 in other populations.



CKD (Lonnemann, 2003) Transplant (Hall et al) Critically Ill (Siew et al)
Perioperative (Parikh et al)
Nephrotoxic (Malyszko, 2009) Hospitalized (Hall et al.) ADPKD (Parikh et al)

Note: Data reported for HIV and HRS cohort is unpublished. For normal, we assumed cardiac surgery patients's preoperative values will be closest to normal individuals.

3.3.5 Correlation among biomarkers of kidney injury

Table 22 presents a correlation matrix for the four biomarkers of kidney injury (ACR, NGAL, NAG, and IL-18). These analyses describe the relationship between each pair of markers but do not control for other variables such as age and sex. Except for the pair ACR-NAG, biomarkers of kidney injury were positively associated with each other.

Table 22. Spearman correlations biomarkers of kidney injury in adolescents (n=200)

	ACR	NGAL	NAG	IL-18
ACR	r=1	r=0.245*	r=0.092	r=0.206*
NGAL		r=1	r=0.126 ***	r=0.537*
NAG			r=1	r=0.147**
IL-18				r=1

* Correlation is significant at the 0.01 level (2-tailed)
** Correlation is significant at the 0.05 level (2-tailed)
*** Correlation is marginally significant at 0.076 level (2-tailed)

We were also interested to know if the same population that had higher values of one biomarker, had higher values of the other biomarkers as well. Table 23 shows that, taking the 75th percentile as a cutoff point for each of the biomarkers, 33% of the population had one biomarker above P75, 20% had two, 7% three, and 1% all four biomarkers.

Table 23. Population with biomarkers above the P75 level.

# biomarkers above P75	n (%)
0	78 (39%)
1	66 (33%)
2	40 (20%)
3	14 (7%)
4	2 (1%)

3.3 Analyses of self-reported symptoms

As explained in the Methods section, participating adolescents completed a questionnaire that included a survey of health problems in general and the frequency of pain while urinating specifically (Appendix 2). Parents also responded whether their child had ever been hospitalized and the reason (Appendix 1). Both children and parents' responses regarding "kidney problems" were noted. Results are shown in Table 24.

A total of 23 parents reported their child (12 boys and 11 girls) to suffer from kidney problems, with no significant differences by school (p=0.967). ¨Kidney" related reasons for hospitalization reported by parents included: "kidney infections" (17), "urinary problems" (2), urinary tract infections (1), "kidney problems" (1), "kidney stones" (1), and "kidney insufficiency" (1). A total of 15 children (10 girls and 5 boys) reported kidney problems, with no significant differences by school (p=0.269). In particular they used terms such as: "kidney infection" (5), "kidney problems" (4), "Pain or burning while urinating" (4), and "kidney pain" (2).

Table 24. Reported symptoms among adolescents

Schools	1. Jinotega	2. Masaya	3. North Chichigalpa	4. South Chichigalpa	TOTAL
	n=63	n=52	n=63	n=67	N=245
	n (%)	n (%)	n (%)	n (%)	n (%)
Symptoms					
Pain while urinating					
Never	38 (60%)	21 (40%)	30 (48%)	44 (66%)	133 (54%)
1-2/y	19 (30%)	19 (37%)	17 (27%)	13 (19%)	68 (28%)
1-2/m	5 (8%)	8 (15%)	11 (18%)	5 (7.5%)	29 (12%)
1 week	1 (2%)	4 (8%)	4 (7%)	5 (7.5%)	14 (6%)
Reported kidney problems	7 (11%)	8 (15%)	9 (14%)	8 (12%)	32 13%)
Reported by child	4 (6%)	6 (12%)	2 (3%)	3 (4%)	15 (6%)
Reported by parent	6 (10%)	5 (10%)	7 (11%)	5 (7%)	23 (9%)

Urine biomarkers of kidney injury were not associated with reported kidney or urinary problems by either parents or children (Table 25).

Table 25. Analysis of biomarkers* by reported kidney and/or urinary problems

	Mean Ratio	LCL	UCL
ACR (ug/mg Crea)	1.00	0.69	1.46
NGAL (ng/mg Crea)	1.09	0.61	1.38
NAG (mU/mg Crea)	1.03	0.68	1.55
IL-18 (pg/mg Crea)	0.99	0.65	1.50

*Biomarkers were Ln-transformed. LCL: Low confidence interval level. UCL: Upper confidence interval level.

The proportion of students who reported "suffering from pain while urinating" frequently (1-2 times per month or weekly), were 10% in School 1, 23% in School 2, 24% in School 3 and 15% in School 4. We further evaluated whether self-reported frequent pain when urinating was associated with urinary markers of early damage, while controlling for age and sex (Table 26). Students with frequent dysuria had a lower concentration of ACR than those with no or infrequent dysuria. There was no association with any of the other biomarkers.

Table 26. Analysis of biomarkers* by frequent dysuria

	Mean Ratio	LCL	UCL
ACR (ug/mg Crea)	0.75	0.53	1.07
NGAL (ng/mg Crea)	0.95	0.72	1.27
NAG (mU/mg Crea)	0.98	0.67	1.44
IL-18 (pg/mg Crea)	1.11	0.81	1.53

*Biomarkers were Ln-transformed LCL: Lower confidence interval level UCL: Upper confidence interval level

IV. CONCLUSIONS

In October and November 2011, the Boston University team conducted an investigation of biological markers of kidney injury among adolescent students at four schools in different regions of Nicaragua (Jinotega, Masaya, and two schools in Chichigalpa). The regions and schools were selected to represent a range of conditions that might affect the risk of developing early-stage kidney damage, which could eventually lead to CKD.

The study was motivated by the frequent diagnosis of CKD among young adults, particularly males, between the ages of 20 and 30, a phenomenon that is quite rare in countries such as the United States. Given that it typically takes many years for damage to the kidney to accumulate to the extent that a person develops clinically apparent CKD, the occurrence of these cases in very young adults raises the question of whether injury to the kidney may actually begin during childhood. Aside from its obvious public health consequences, the answer to this question has important implications for the search for causes of the CKD epidemic, since identification of early-stage kidney damage among children would highlight the need to study factors that could affect children before they enter the workforce.

The specific objectives of this study were:

1) To assess whether there is any evidence of early-stage kidney damage among adolescents in different areas of Nicaragua chosen to represent a range of demographic and environmental conditions and thus potential risk for kidney damage; and
2) To assess whether there are differences in the prevalence of early-stage kidney damage by sex, age, and school consistent with the patterns of the CKD epidemic among adults.

Before summarizing the specific findings, we wish to note that a study such as this one, in which apparently healthy adolescents are tested in a community setting using novel biomarkers of kidney injury is unique. We are not aware of any other studies of its kind. The results of the study are provocative, yet at the same time puzzling in some ways. Owing to the uniqueness of the study, as well as its relatively small size, we believe it is important to consider these results as preliminary and avoid drawing firm conclusions until we and/or other researchers have had a chance to replicate them and further consider issues that may have arisen in the design, implementation, or analysis of the study.

- **Is there evidence of early-stage kidney damage among adolescents in Nicaragua?**

  The results of our study indicate that there may indeed be early-stage kidney damage among adolescents in Nicaragua. There are two main pieces of evidence for this conclusion:

  i) The average level of the kidney biomarker IL-18 in this population of children was higher at all schools than in healthy participants in studies in other countries.
  ii) Certain groups in the study had concentrations of different biomarkers of kidney damage that were elevated compared to other groups. This evidence is discussed in more detail in the next section of the Conclusions.

  The albumin-creatinine ratio (ACR) biomarker has an accepted level above which kidney damage is considered to be present, whereas the other three biomarkers evaluated in this study (NGAL, NAG, and IL-18) do not. All three of this latter group of biomarkers have been more recently introduced, and the scientific community is still learning about them. One of their main strengths, and the reason we used them in this study, is their ability to detect kidney damage before it becomes severe enough to meet the clinical definition of CKD by measurements of serum creatinine. We did not expect that any students would have progressed to CKD and so wanted to use biomarkers that are sensitive to detect early-stage kidney damage. We also did not want to use the more invasive method of collecting blood samples that would be necessary to evaluate serum creatinine.

  Because of the lack of a cutoff value for kidney damage for these three markers, we compared the concentrations among Nicaraguan adolescents to those reported in other studies, which included both individuals with kidney conditions and those who were apparently free of kidney damage ("normal controls"). We found that the results in our study were similar to normal controls for NGAL and NAG, but were higher than expected for IL-18. IL-18, along with NGAL and NAG, is a biomarker of damage to the renal tubules. This is significant because it suggests that damage would be occurring in this area of the kidney, which is consistent with the strong evidence that CKD in young adults in Nicaragua is of the tubulointerstitial type. Adding to the evidence that damage would be focused in the tubules, few students had elevated levels of ACR, which is primarily a biomarker of damage to the glomerulus.

- **Are there differences in early-stage kidney damage among adolescents in Nicaragua consistent with the patterns of the CKD epidemic among adults?**

  The epidemic of CKD among adults is occurring primarily among men who work as manual laborers, in Western Nicaragua, and among residents at lower altitude. Based on these patterns, we hypothesized that the frequency of early-stage kidney damage would be higher

among boys and would vary by school in the following order, ranked from lowest to highest: Jinotega, Masaya, north Chichigalpa, south Chichigalpa.

Contrary to our expectations, girls had higher levels than boys for all biomarkers, with particularly large differences for ACR and IL-18. We also found that girls had higher urine specific gravity levels than boys, which indicates that their urine is more concentrated. The increased concentration is not in and of itself a sign of kidney damage, and represents relative dehydration which may simply be from fluid restriction due to the greater propensity for girls to avoid using the toilet in a public setting. However, this does not seem to explain the difference in biomarker levels between girls and boys, since the same relative relationship was seen whether or not we accounted for urine creatinine.

We do not have an explanation for the finding of higher levels of biomarkers in girls than boys. However, this result does not necessarily contradict the male predominance of CKD observed in adults. We have in the past proposed that it is likely that more than one factor must be responsible to explain such a large excess of CKD in the region, and that these factors could interact synergistically. For example, one factor might make the kidney more susceptible to damage from a second factor, or one factor might initiate damage, while another speeds up its progression. According to this model, it is possible that women are somewhat more affected by some factor that affects children but are less likely to go on to develop CKD because they are less likely to be heavily exposed to the second factor. Admittedly, this is quite speculative, and the finding of higher biomarker concentrations among girls remains puzzling.

Results according to school were more in accordance with our *a priori* hypothesis. The results for NAG were most consistent in this regard, with the relative concentrations rank ordered by school according to risk profile (Jinotega, Masaya, north Chichigalpa, south Chichigalpa) in both boys and girls. Boys at the school in south Chichigalpa, which we had hypothesized would have the highest prevalence of kidney damage, had elevated levels of all three tubular biomarkers—NGAL, NAG, and IL-18—compared to boys at the school in Jinotega. Both boys and girls at both of the two schools in Chichigalpa had elevated levels of NAG compared to children at the school in Jinotega. Thus, both schools in Chichigalpa demonstrated some evidence of kidney damage among both boys and girls, but only boys at the school in south Chichigalpa exhibited the most consistent evidence across all three tubular biomarkers. Again, it is notable that the biomarker ACR, which is primarily associated with glomerular damage, did not demonstrate these patterns.

School itself is a proxy for other exposures associated with residence. Because it has been reported that many adolescents in the past began working at a young age and in some this may still be the case, it has been suggested that kidney damage in this age group would

simply reflect exposure to occupational factors. However, we excluded students who self-reported any significant work history.

Overall, our results suggest some evidence of early-stage kidney damage in adolescents in Nicaragua that appears to extend to all regions studied, but with higher frequency in Chichigalpa. This finding is preliminary and requires further data collection and examination. If the finding is correct, it will be important to identify the causes and the relationship to the ongoing epidemic of CKD in adults. Some of the factors that could be investigated include direct or indirect (due to contaminants brought home on parents' clothing) occupational exposure, environmental exposures, infectious diseases, low birth weight and genetic susceptibility. More insight into the possible role of these factors would have implications not only for the health of children but also for the causes of the CKD epidemic in Nicaragua and throughout Central America.

Although we elected to focus on adolescents because they were young enough that we could capture them before they began working yet old enough to exhibit kidney damage if it had occurred, it would be important to test younger children to see whether any signs of kidney damage can be detected at an even earlier age. One such study was conducted in 1998 in the city of León among 423 pre-school children (3-6 years old) (Pastora Coca, 1998). Using urine dipsticks, they found 51.4% prevalence of some level of proteinuria and 20.3% of hematuria. These percentages are much higher than the urine dipstick results in our study. In fact, had we used only urine dipstick, little evidence of damage would have emerged. Consideration should also be given to collecting blood as well as urine in future studies. As a result, we were not able to test for any biological markers in blood, such as serum creatinine. Studies in other countries in the region that are also experiencing the epidemic would also be worthwhile.

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APPENDIX 1

[PARENT QUESTIONNAIRE]

Please answer the following questions:

1. How old is your child who brought home the information about the study by Boston University?

2. Has this child ever been hospitalized for any reason? Yes ☐ No ☐

For each hospitalization, please list the reason and the child

3. How many years have you lived at your current address?

4. How many years have you lived in [Chichigalpa, Jinotega, Masaya]

	Father	Mother
Is this person alive or dead?	Yes ☐ No ☐	Yes ☐ No ☐
If dead, what was the cause of death?		
Highest grade completed in school		
Have you suffered or do you now suffer from any of these illnesses?	Chronic Renal Insufficiency Yes ☐ No ☐ Kidney stones Yes ☐ No	Chronic Renal Insufficiency Yes ☐ No ☐ Kidney stones Yes ☐ No ☐ Other Kidney Problem Yes ☐ No ☐

	☐ Other Kidney Problem Yes ☐ No ☐ ☐ High Blood Pressure Yes ☐ No ☐ Diabetes/High Blood Sugar Yes ☐ No ☐	High Blood Pressure Yes ☐ No ☐ Diabetes/High Blood Sugar Yes ☐ No ☐
Ever work in sugar cane?	Yes ☐ No ☐ Number of years: _____	Yes ☐ No ☐ Number of years: _____
What is your current occupation?	Number of years: _____	Number of years: _____
What occupation have you worked in the longest?	Number of years: _____	Number of years: _____

APPENDIX 2

[CHILD QUESTIONNAIRE]

1. Sex

2. Age

3. Have you ever worked to help out your family? Yes ☐ No ☐

How many months or years did you work to help your family?

What kind of work did you do?

Are you working to help your family now?

4. Other than helping your family, have you ever worked at a job for which you were paid?

Yes ☐ No ☐

How many months or years did you work at this job?

What kind of place did you work at? (ex., fields, store, factory, office)

What kind of work did you do?

Are you working at a job now?

5. Have you done any sports or vigorous exercise today? Yes ☐ No ☐

6. About how long did it take you to walk to school today?

7. Do you have any ongoing problems with your health? Yes ☐ No ☐

What kinds of problems?

8. How often do you have pain when you urinate?

Never ☐ 1-2 times a year ☐ 1-2 times a month ☐ Once a week or more ☐