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Evaluation of kidney injury biomarkers in an adult Mexican population environmentally exposed to fluoride and low arsenic levels

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ABSTRACT

Fluoride (F) is a toxicant widely distributed in the environment. Experimental studies have shown kidney toxicity from F exposure. However, co-exposure to arsenic (As) has not been considered, and epidemiological information remains limited. We evaluated the association between F exposure and urinary kidney injury biomarkers and assessed As co-exposure interactions. A cross-sectional study was conducted in 239 adults (18–77 years old) from three communities in Chihuahua, Mexico. Exposure to F was assessed in urine and drinking water, and As in urine samples. We evaluated the urinary concentrations of albumin (ALB), cystatin-C (Cys-C), kidney injury molecule 1 (KIM-1), clusterin (CLU), osteopontin (OPN), and trefoil factor 3 (TFF-3). The estimated glomerular filtration rate (eGFR) was calculated using serum creatinine (Creat) levels. We observed a positive correlation between water and urine F concentrations ($\rho = 0.7419$, $p < 0.0001$), with median values of 1.5 mg/L and 2 μg/mL, respectively, suggesting that drinking water was the main source of F exposure. The geometric mean of urinary As was 18.55 ng/mL, approximately 39% of the urine samples had As concentrations above the human biomonitoring value (15 ng/mL). Multiple linear regression models demonstrated a positive association between urinary F and ALB ($\beta = 0.56$, $p < 0.001$), Cys-C ($\beta = 0.022$, $p = 0.001$), KIM-1 ($\beta = 0.048$, $p = 0.008$), OPN ($\beta = 0.38$, $p = 0.041$), and eGFR ($\beta = 0.49$, $p = 0.03$); however, CLU ($\beta = 0.07$, $p = 0.100$) and TFF-3 ($\beta = 1.14$, $p = 0.115$) did not show significant associations. No interaction with As exposure was observed. In conclusion, F exposure was related to the urinary excretion of early kidney injury biomarkers, supporting the hypothesis of the nephrotoxic role of F exposure.

1. Introduction

Inorganic fluoride (F) is a toxicant widely distributed in the environment (air, soils, rocks and water). Naturally high F concentrations can occur in groundwater sources, depending on the geological setting, hydrological conditions and the presence of fluoride-bearing minerals in the rocks and soil (Jha et al., 2013). Therefore, drinking water is recognized as a major contributor to human F exposure. In many countries and in northern-central regions of Mexico, drinking water exceeds the F concentration of 1.5 mg/L established by the World Health Organization as guideline value for human drinking water (WHO, 2017). An estimate of > 200 million people worldwide exposed to high F levels through drinking water has been reported (Edmunds and Smedley, 2013).

The F contribution to the prevention of dental caries is recognized. Nevertheless, exposure to high F levels has been associated with adverse effects such as dental and skeletal fluorosis, characterized by dental enamel hypomineralization and brittle bones, as well as reductions in cognitive functions and decreases in male fertility and thyroid dysfunction (Choi et al., 2015; Izquierdo-Vega et al., 2008; NRC, 2006; Singh et al., 2014). Exposure to F is linked to broad toxicity mechanisms such as enzyme inhibition, induction of apoptosis, cell cycle arrest and oxidative stress, some of which are also recognized as mechanisms associated with chronic kidney damage (Barbier et al., 2010; Ozbek, 2012).

The kidneys are the main route of F removal from the body, and

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approximately 60% of the total daily F absorbed is filtered and excreted in urine (Buzalaf and Whitford, 2011). The kidneys can concentrate F as much as 50-fold from plasma to urine, making kidney cells susceptible to damage (NRC, 2006). Experimental studies have clearly shown nephrotoxic effects of F exposure. Renal histological changes, major toxic effects in the S3 segment of the proximal tubule (PT), oxidative stress generation, mitochondrial impairment by the sirtuin 3 (SIRT3) pathway and apoptosis induction via activation of Bax expression and Bcl-2 suppression have been reported (Song et al., 2017; Usuda et al., 1998; Xiong et al., 2007; Xu et al., 2006; Zhan et al., 2006). Further, ecological studies show elevated F content in drinking water in high chronic kidney disease (CKD) prevalence zones, suggesting a possible link between excess F in drinking water and local foods, and kidney disease (Chandrajith et al., 2011; Dharmaratne, 2015). However, epidemiological information remains limited due in part to the lack of sensitive biomarkers to detect early kidney toxicity.

In the kidney damage processes, several pathophysiological process and molecules are involved that can be used as early kidney injury biomarkers, which are more sensitive and specific than traditional biomarkers [serum creatinine (Creat) and blood urea nitrogen (BUN)] (Bonventre et al., 2010; Vaidya et al., 2008). Previous studies have reported increased urinary excretion of the early kidney injury biomarkers: kidney injury molecule 1 (KIM-1), clusterin (CLU), osteopontin (OPN), heat shock protein 72, cystatin-C (Cys-C), and β2-microglobulin in rats sub-chronically exposed to relevant F levels to human exposure, suggesting a tubular dysfunction due to F exposure (Cárdenas-González et al., 2013). Additionally, increased urinary levels of *N*-acetyl-β-glucosaminidase (NAG) and γ-glutamyl transpeptidase (γ-GT) have been reported in children, who drink water with F levels above 2 ppm. However, either NAG or γ-GT are specific to kidney damage, and no others risk factors were considered in the analyses (Xiong et al., 2007). Furthermore, co-exposure to other potential nephrotoxicants, such as arsenic (As), that commonly co-occur with F in drinking water has also not been considered (González-Horta et al., 2015). Few studies have focused on assessing F nephrotoxicity using early kidney injury biomarkers, the epidemiological information about the nephrotoxicity of F exposure is not conclusive, and the effect(s) of F-As co-exposure in kidney is even more limited.

Therefore, the aim of this study was to evaluate the urinary concentration of early kidney injury biomarkers albumin (ALB), Cys-C, OPN, CLU, KIM-1 and trefoil factor 3 (TFF-3) in an adult population exposed to F in drinking water and evaluate the effect of co-exposure to relative low As levels.

2. Materials and methods

2.1. Study population

We performed a cross-sectional study in 239 adult residents of three municipalities in Chihuahua, México in July 2013 (Fig. 1). This study was approved by the Institutional Bioethics Committee for Research in Humans (COBISH-CINVESTAV). The population was invited to participate through community boards, coordinated by the local authorities. The inclusion criteria consisted of adults (> 18 years old) who commonly drink tap water, and a minimum of 1 year of residence in the study area. A drinking water sample was provided by each participant. Adults who reported cancer or kidney disease were excluded from the study. Written informed consent was obtained from all individual participants included in the study. A structured questionnaire was used to determine general characteristics of the participants, sources of drinking water, health history, smoking exposure, medication, and some non-specific symptoms.

	Community	F tap water	As tap water
●	El Sauz	0.2 mg/L	0.01 μg/L
●	Aldama	2.0 mg/L	9.7 μg/L
●	Gpe. Victoria	5.2 mg/L	41.6 μg/L

Fig. 1. Location of the communities included in the study in Chihuahua, Mexico. The values of the concentration of F and As in drinking water determined in previous monitoring are presented in the table, which were used for the selection of the study communities.

2.2. Participants general health examinations and serum biochemical analyses

We measured height using standard protocols. The body weight and body mass index (BMI) was calculated using an impedance bioanalyzer (InBody230, Biospace Co., Ltd., Korea), and the population were classified according to WHO's body mass index classification as underweight (< 18), normal (18 to < 25), overweight (≥25 to < 30) and obese (≥30) (WHO, 2006). The blood pressure measurements were conducted by a physician using a calibrated sphygmomanometer in sitting position. For the analysis of serum Creat, lipids, glucose and alkaline phosphatase, blood samples were obtained using sealed separation gel tubes (Becton Dickinson, USA) and centrifuged at 1500 rpm for 15 min; the supernatant was aliquoted and stored at 4 °C until biochemical analysis using an automatic analyzer (Prestige 24i, Tokyo Boeki Medical System Ltd., Tokyo). The estimated glomerular filtration rate (eGFR) was determined by the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) formula (Levey et al., 2009) using serum Creat concentration. The atherogenic index of plasma was calculated as log (triglycerides/high density lipoproteins) in molar concentrations (Dobiášová and Frohlich, 2001). Dental fluorosis was evaluated by qualified odontologist using Dean's Classification System for Dental Fluorosis (Dean, 1942).

2.3. Urine sample collection and urinary kidney damage biomarkers assay

A first morning void urine sample was provided by each participant. Urinalysis was performed immediately by urinalysis strips (U-11 Urinalysis Reagent Strips, Mindray Co., China). A 2× protease inhibitor

solution (Sigma FASTTM, Sigma-Aldrich Corp., USA) was mixed with an aliquot of the urine sample (1:3000) to prevent the urinary proteolysis. After centrifugation at 3000 rpm for 10 min, the urine supernatant was aliquoted into polypropylene tubes, transported and stored at −80 °C until analysis, the urinary sediment was resuspended and manual microscopic sediment analysis for detection of urine formed elements, specially erythrocytes, leucocytes, epithelial cells, mucoprotein, bacteria, yeast, casts and crystals (uric acid, calcium oxalate, amorphous urates and amorphous phosphates) was performed by a qualified analyst. Long periods of storage (> 6 months) or multiple (> 2) freeze/thaw cycles were avoided before the analysis. Urinary albumin (ALB), cystatin-C (Cys-C), osteopontin (OPN), clusterin (CLU), trefoil factor 3 (TFF-3) and kidney injury molecule 1 (KIM-1) levels were determined by Luminex xMAP® Technology (Millipore Corp., USA) using MILLIPLEX® MAP Human Kidney Toxicity panel 3 and panel 4 following the manufacturer's instructions. Before the analysis was performed, urine samples were thawed and centrifuged at 3000 rpm for 10 min at 4 °C, and the supernatant was used for analysis. High and low concentration controls were also included in each plate. Urinary Creat concentration was assessed using a commercial kit based on the Jaffe reaction (Creat, Randox Laboratories Ltd., UK). All samples were analyzed in duplicate. The urinary concentration of early kidney injury biomarkers was adjusted by specific gravity and Creat.

2.4. Fluoride and arsenic measurements

The concentration of F in water and urine samples was assessed by a potentiometric method using an ion selective electrode (Orion 9609BNWP, Thermo Fisher Scientific Inc., USA), where equal volumes of the sample and total ionic strength adjustment buffer (TISAB) were mixed, and the F concentration was determined by an interpolation of the potential results in a calibration graph (Del Razo et al., 1993). The analysis of urine fluoride reference material (PC-U-F1114, Quebec Centre of Toxicologie, level 1.38 μg/mL) was used as a quality control. The accuracy obtained ranged from 95 to 103%, and the coefficients of variation for duplicate samples were lower than 7%.

The urine concentration of inorganic arsenic and its metabolites [monomethylarsonic acid (MAs) or dimethylarsinic acid (DMAs)] were assessed by hydride generation-cryotrapping-atomic absorption spectrometry using a Perkin Elmer Analyst 400 spectrometer (Perkin Elmer, Norwalk, CT) equipped with a multi-atomizer (Hernández-Zavala et al., 2008). The results are expressed as a total urinary arsenic (tAs) value, which is the sum of the levels of inorganic arsenic and its metabolites. We used standard reference material (SRM) 2669 level 1 and level 2 from the National Institute of Standard and Technology (NIST) to validate the analysis of the arsenic species at low and high concentrations in urine, respectively. Repeated analysis of SRM2669 indicated coefficients of variation lower than 11% and an accuracy of 97–103% for high and low tAs values.

Urine tAs and F concentrations were normalized by the Levine-Fahy method using urinary strip specific gravity (Levine and Fahy, 1946).

2.5. Statistical analysis

We performed exploratory analysis to assess the data quality and the distribution of the variables of interest. All continuous variables are described as the mean ± standard deviation (SD) or geometric mean (interquartile range). Categorical variables are reported as frequencies or percentages. Spearman's correlation test was performed to assess the relationship between water and urine F concentrations. All statistical analyses of urinary kidney injury biomarkers were performed without normalization, normalized by specific gravity and normalized by urinary Creat. Robust multiple linear regression analysis was performed for each kidney injury biomarker. The results are expressed as the regression coefficient (β) and p-value for each explanatory variable. The co-variates were selected based on biological plausibility and improved model fit (contribution to R^2-adjusted value and/or at least 10% of change of regression coefficient value). Co-linearity, as variance inflation factor, was reviewed for all regression models. Additionally, an interaction analysis was performed to assess the possible interaction between urinary F and tAs as a continuous and dichotomized (15 ng/mL as cut off value) variable for all regression models. All analyses were performed using STATA version 14.0 (Stata Corp., USA), and p-values < .05 were considered statistically significant; in addition, p-values < .1 were considered marginally statistically significant.

Table 1
General characteristics of the study population.

Parameter	n	Mean ± SD, GM (P25-P75) or percentage
Sociodemographic variables		
Community		
El Sauz	106	45
Aldama	84	36
Guadalupe Victoria	46	19
Age (years)	236	46.1 ± 13.3
Sex		
Males	68	28.8
Females	168	71.2
Occupation		
Homemaking	147	61
Laborer	24	10
Merchant	9	4
Other	59	25
Education		
Illiterate	51	21.4
Elementary	85	35.7
Middle	69	29.0
High School or Technical	29	12.2
Graduate	3	1.3
Postgraduate	1	0.4
Anthropometric		
BMI (kg/m^2)	233	29 (26–33)
Normal	42	18
Over-weight	81	35
Obese	110	47
Biochemical analysis		
Glucose (mg/dL)	235	93 (87–103)
≥126 mg/dL	27	11.5
Triglycerides (mg/dL)	235	201 ± 137
High (> 200 mg/dL)	91	39
Total cholesterol (mg/dL)	235	194 ± 43
High (> 240 mg/dL)	39	17
LDL cholesterol (mg/dL)	235	115 ± 77
HDL cholesterol	235	47 ± 9
Alkaline phosphatase (UI)	235	298 (253–371)
Diabetes	30	12.7
Hypertension	67	28.4
DBP (mmHg)	233	79 (70–87)
SBP (mmHg)	233	127 (116–143)
Smoking	48	20.1

Results are expressed as the mean ± standard deviation, percentage or geometric mean (P25-P75). Abbreviations: BMI, body mass index; LDL, low density lipoproteins; HDL, high density lipoproteins; SBP, systolic blood pressure; DBP, diastolic blood pressure; P, percentile.

3. Results

3.1. Characteristics of the study population

From 239 participants, we excluded 3 with no urine samples available. Table 1 describes the sociodemographic, anthropometric, biochemical and health status characteristics of the study population. Of the total population, 71.2% (n = 168) were females, and the mean age ± SD of the participants was 46 ± 13 years old, ranging from 18 to 77 years. Homemaking was the main occupation for females and laborer for males, and 86.3% of the population had a middle school education degree or lower. The median BMI was 29 kg/m^2, and 47% of

Table 2
Arsenic and fluoride exposure of the study population.

Parameter	n	GM (IQR) or percentage
Fluoride		
Water (mg/L)	232	1.5 (0.19–1.8)
≥1.5 mg/L	118	50.9
Urine (μg/mL)	236	2.0 (1.1–3.5)
≥1.6 μg/mL[a]	140	59.3
Dental fluorosis		
Normal	68	31.78
Questionable	25	11.68
Very mild	39	18.22
Mild	42	19.63
Moderate	26	12.15
Severe	14	6.54
Urine As analysis		
Total As (ng/mL)	236	18.55 (10.6–34.1)
≥15 ng/mL[b]	139	58.9
Inorganic As (ng/mL)	236	1.8 (0.91–4.4)
MAs (ng/mL)	236	1.8 (0.19–4.7)
DMAs (ng/mL)	236	12.8 (6.7–26.4)

Abbreviations: GM, geometric mean; IQR, interquartile range (P25-P75); As, arsenic; MAs, monomethylarsonic acid; DMAs, dimethylarsinic acid.

[a] Biomonitoring Equivalent value for urinary fluoride that corresponds to the Minimum Risk Level from ATSDR for the adult population (Aylward et al., 2015).

[b] Human biomonitoring value derived by the German Human Biomonitoring Commission, reference value (RV_{95}) for total urinary arsenic (Schulz et al., 2011).

the population was obese (BMI ≥ 30 kg/m^2). The mean concentrations of glucose, total cholesterol, LDL cholesterol, triglycerides, and HDL cholesterol were classified as normal. However, 11.5% of the population showed fasting glucose concentrations above the diabetes diagnostic cut-off point (ADA, 2013), 39% showed hypertriglyceridemia, and 17% showed hypercholesterolemia. Of the total population, 12.7% self-reported diabetes, and 28.4%, hypertension. The tobacco smoking prevalence was 20%, and the mean tobacco smoking index in this group was 3.6. The urinalysis characteristics are summarized in Supplementary materials Table S1. A positive correlation was observed between strip specific gravity and urinary Creat (Spearman's coefficient = 0.6794, $p < .0001$).

3.2. Fluoride exposure assessment

Water and urine F concentrations and dental fluorosis results are summarized in Table 2. Drinking water samples had a median F concentration of 1.5 mg/L, ranging from 0.1–5.1 mg/L, and 51% had concentrations above the maximum permissible limit of F (1.5 mg/L) in drinking water. The median urinary F concentration was 2 μg/mL, ranging from 0.4 to 27 μg/mL, and approximately 59% of the urine samples had F concentrations ≥1.6 μg/mL, the Biomonitoring Equivalent (BE) value of urinary F that corresponds to the Minimal Risk Level from ATSDR for adult populations (Aylward et al., 2015). Dental fluorosis, a chronic exposure biomarker, had a prevalence of 57% (very mild to severe grades). However, the evaluation was not possible in 22 participants owing to an absence teeth or highest dental wear. In addition, the results showed a positive and significant correlation between F concentration in water and urine (Spearman's coefficient = 0.7419, $p < .0001$) as well a relationship with dental fluorosis grade ($p < .0001$) (Fig. 2). Further, both urinary and water F concentrations increased with increasing dental fluorosis grade (Table S2).

3.3. Arsenic exposure assessment

Arsenic speciation results are summarized in Table 2. tAs is the urinary sum of the inorganic and arsenic species (MAs and DMAs). In the population, the tAs concentration median was 18.5 ng/mL, and 59% of the samples had concentrations above the reference value (RV_{95}, 15 ng/mL) of the German Human Biomonitoring Commission, which was derived from the 95% confidence interval of the 95th population percentile of the concentration level of urinary tAs in a German population who did not eat fish prior to the urine collection (Schulz et al., 2011).

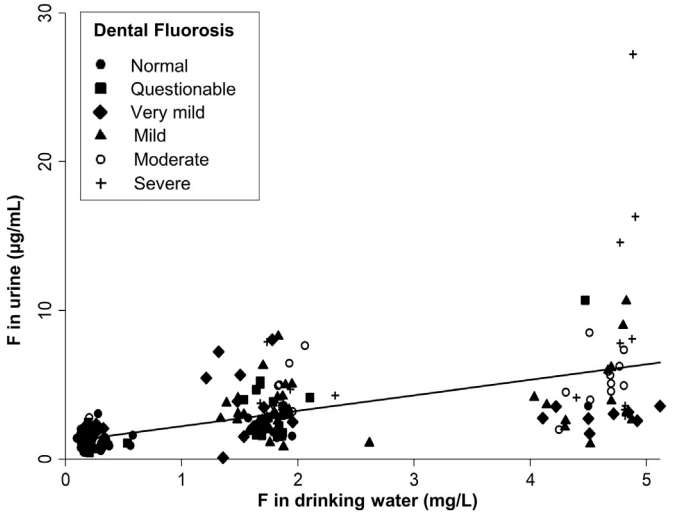

Fig. 2. Relationship between urinary and water fluoride concentrations in the study population. A Spearman's correlation ($\rho = 0.7419$, $p < 0.0001$, $n = 232$) and dental fluorosis stage denoted by symbols are showed.

3.4. Kidney injury biomarkers and F exposure associations

ALB, Cys-C, OPN, CLU, TFF-3 and KIM-1 are the biomarkers that we decided to measure because of their high sensitivity and, for some of them, specificity in the early detection of kidney injury (Table S3).

Table 3 shows the levels of traditional and novel kidney injury biomarkers in the study population. Most of the urine samples contained detectable amount of the kidney biomarkers with exception of a proportion of 2.1 (n = 5) and 0.2 (n = 1) for KIM-1 and TFF-3 samples, respectively that were below the limit of detection (LOD), which were substituted by LOD/√2. The traditional biomarkers serum Creat and BUN had a mean concentration of 1.14 ± 0.14 mg/dL and 13.2 ± 3.8 mg/dL, respectively. The serum Creat values were used to

Table 3
Serum biomarkers, filtration rate and urinary kidney biomarkers levels in the study population.

Biomarker	n	Mean ± SD	GM	Range
Serum				
Creatinine (mg/dL)				
Men	67	1.26 ± 0.12	1.3	1.1–1.6
Female	168	1.09 ± 0.12	1.1	0.8–1.6
BUN (mg/dL)	235	13.2 ± 3.8	12.6	4.6–28.5
eGFR CKD-EPI (mL/min/1.73 m^2)	235	64 ± 10	64	33–101
Urine				
Albumin (μg/mL)[a]	234	9.9 ± 9.1	7.19	0.66–65.9
Clusterin (μg/mL)[a]	236	0.91 ± 1.0	0.69	0.06–10.2
Cystatin-C (μg/mL)[a]	236	0.20 ± 0.18	0.15	0.013–1.6
Osteopontin (μg/mL)[a]	236	1.12 ± 0.89	1.00	0.03–9.7
KIM-1(ng/mL)[a]	235	0.90 ± 0.71	0.66	0.008–3.2
TFF-3 (ng/mL)[a]	236	13.3 ± 18.3	9.1	0.23–170.8

Abbreviations: SD, standard deviation; GM, geometric mean; BUN, blood urea nitrogen; eGFR estimated glomerular filtration rate; CDK-EPI, Chronic Kidney Disease Epidemiology Collaboration; KIM-1, kidney injury molecule 1; TFF-3, trefoil factor 3.

[a] Normalized by urinary specific gravity.

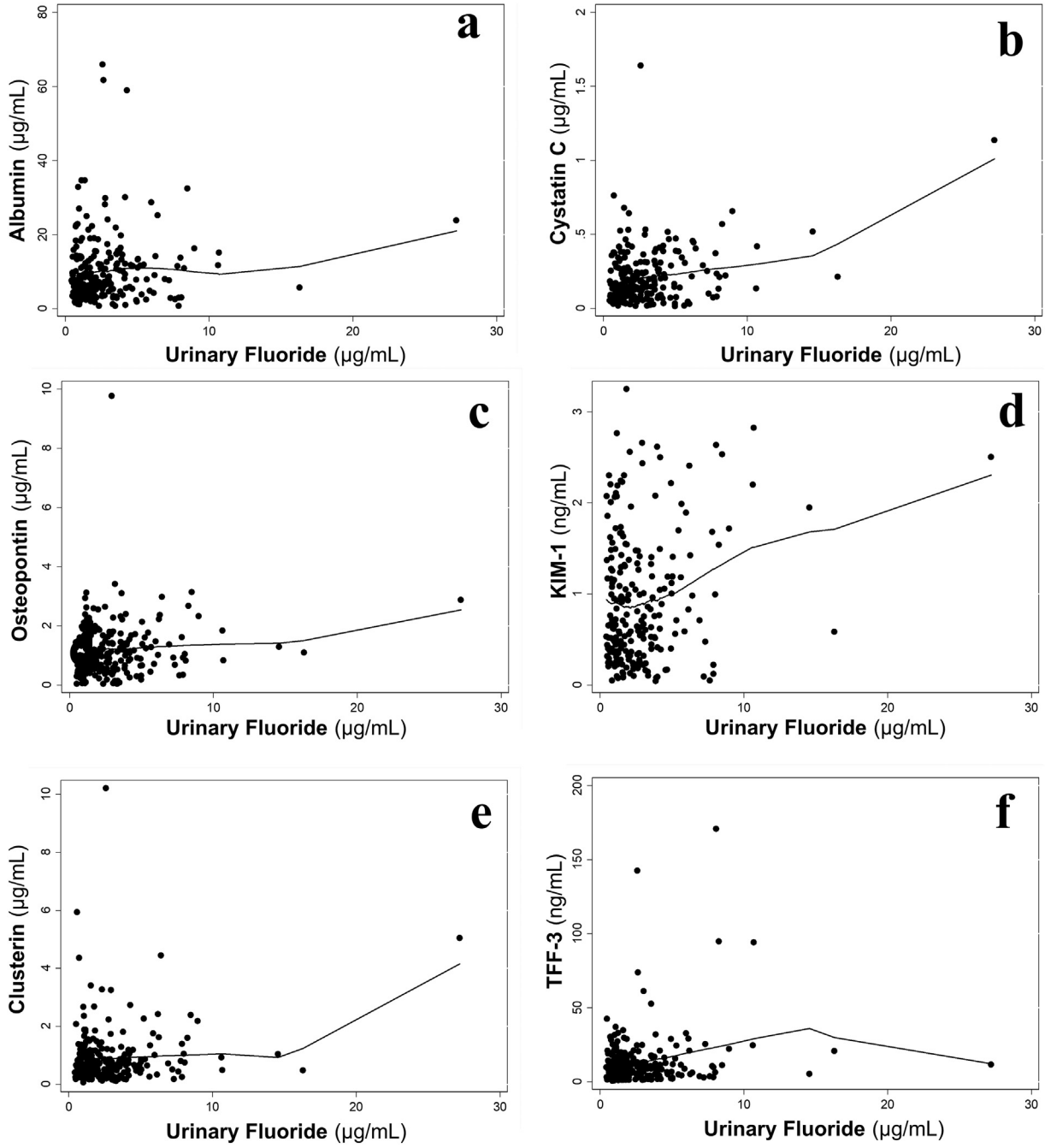

Fig. 3. Relationship between urinary and water fluoride concentrations and the urinary concentrations of (a) albumin, (b) cystatin-C, (c) osteopontin, (d) KIM-1, (e) CLU and (f) TFF-3, with superimposed smoothed plots (LOWESS smoothing bandwidth = 0.8).

estimate the eGFR, a marker of kidney function, and the results showed a mean eGFR of 64 ± 10 mL/min/1.73 m^2 and a moderately reduced kidney function (30–59 mL/min/1.73 m^2) prevalence of 38%. The ALB mean concentration was 9.9 ± 9.1 µg/mL and the microalbuminuria (30–300 mg/g Creat) prevalence was 3% (n = 7).

The smoothed relationship between the urine F concentration and the urinary kidney injury biomarkers are shown in Fig. 3. In a simple regression analysis, positive statistically significant relationships were observed between urine F concentration and urinary Cys-C (β = 0.02 µg/mL, p-value < .001), CLU (β = 0.06 µg/mL, p-value = .006), OPN (β = 0.05 µg/mL, p-value = .019), KIM-1 (β = 0.05 ng/mL, p-value = .001) and TFF-3 (β = 1.1 ng/mL, p-value = .008).

The multiple robust linear regression models performed to evaluate the associations between F exposure and the kidney injury biomarkers are described in Table 4. For all biomarkers, the water and urine F concentration were included in separate models as independent variables, the urinary concentration was normalized by specific gravity. The models for ALB estimated an average increase of 1.19 µg/mL in ALB (β value) per unit increase in water F concentration (p-value < .001) and an average increase of 0.56 µg/mL per unit increase in urinary F concentration (p-value < .001); the models were adjusted by urine protein content, history of mine work, diabetes, urine leucocytes, age and sex. The models for Cys-C estimated an average increase of 0.03 µg/mL in Cys-C per unit increase in water F concentration (p-value = .005) and an average increase of 0.022 µg/mL in Cys-C per unit increase in urinary F concentration (p-value = .001); the models were adjusted by sex, urine protein content and the presence of amorphous

Table 4
Multiple robust linear regression analysis of kidney injury biomarkers normalized by urinary specific gravity and the estimated glomerular filtration rate in the study population.

	Explanatory variables	F in water		F in urine	
		β[a]	p-Value	β[a]	p-Value
Albumin[c] (μg/mL)	F in water (mg/L)	1.20	< 0.001	–	–
	F in urine[b] (μg/mL)	–	–	0.56	< 0.001
	Protein (15 mg/dL)	2.5	0.010	1.51	0.089
	Protein (30 mg/dL)	31.8	< 0.001	31.3	< 0.001
	Mine-worker	8.6	0.029	8.3	0.036
	Diabetes	3.7	0.035	4.2	0.020
	Urine leucocytes (> 5 cell/ field)	3.6	0.008	3.8	0.006
	Age (years)	−0.01	0.769	−0.02	0.487
	Female	−1.7	0.117	−1.52	0.156
Cystatin-C[d] (μg/mL)	F in water (mg/L)	0.03	0.005	–	–
	F in urine[b] (μg/mL)	–	–	0.022	0.001
	Protein (15 mg/dL)	0.08	< 0.001	0.061	0.001
	Protein (30 mg/dL)	0.31	0.105	0.30	0.128
	Amorphous urate crystals	0.04	0.148	0.07	0.027
	Age (years)	−0.0003	0.758	−0.0005	0.590
Osteopontin[e] (μg/mL)	F in water (mg/L)	0.10	0.028	–	–
	F in urine[b] (μg/mL)	–	–	0.038	0.041
	Amorphous urate crystals	0.25	0.239	0.333	0.163
	Age (years)	−0.01	0.009	−0.01	0.007
	Female	−0.27	0.079	−0.256	0.118
Clusterin[f] (μg/mL)	F in water (mg/L)	0.09	0.118	–	–
	F in urine[b] (μg/mL)	–	–	0.07	0.100
	Protein (15 mg/dL)	0.37	0.014	0.31	0.008
	Protein (30 mg/dL)	1.9	0.129	1.89	0.133
	Smoking index	0.03	0.072	0.028	0.165
	Age (years)	0.004	0.448	0.005	0.401
	Female	0.112	0.385	0.122	0.330
KIM-1[g] (ng/mL)	F in water (mg/L)	0.045	0.162	–	–
	F in urine[b] (μg/mL)	–	–	0.048	0.008
	Amorphous urate crystals	0.262	0.065	0.264	0.055
	Mucoprotein	0.459	< 0.001	0.382	0.002
	Atherogenic index	0.233	0.143	0.227	0.138
	Age (years)	−0.004	0.270	−0.004	0.251
TFF-3[h] (ng/mL)	F in water (mg/L)	2.88	0.010	–	–
	F in urine[b] (μg/mL)	–	–	1.14	0.115
	Diabetes	8.9	0.069	8.85	0.068
	Age (years)	−0.086	0.046	−0.15	0.028
	Female	0.86	0.669	0.99	0.631
eGFR[i] (mL/min/1.73 m^2)	F in water (mg/L)	0.19	0.675	–	–
	F in urine[b] (μg/mL)	–	–	0.49	0.030
	Vascular diseases	−4.9	< 0.001	−5.06	< 0.001
	Cholesterol (mg/dL)	−0.05	< 0.001	−0.05	< 0.001
	Alkaline phosphatase (IU)	−0.022	0.003	−0.02	0.005
	Nephrotoxic drugs use	−5.2	0.001	−5.34	0.001

Abbreviations: KIM-1, kidney injury molecule 1; TFF-3, trefoil factor 3; eGFR, estimated glomerular filtration rate.
[a] Average difference of the biomarker per unit change in the explanatory variable.
[b] Adjusted by specific gravity.
[c] F in water R^2-Adjusted = 0.4589, n = 230; F in urine R^2-Adjusted = 0.4467, n = 234.
[d] F in water R^2-Adjusted = 0.1692, n = 232; F in urine R^2-Adjusted = 0.2306, n = 236.
[e] F in water R^2-Adjusted = 0.0944, n = 232; F in urine R^2-Adjusted = 0.0800, n = 236.
[f] F in water R^2-Adjusted = 0.1391, n = 232; F in urine R^2-Adjusted = 0.1550, n = 236.
[g] F in water R^2-adjusted = 0.1088, n = 230; F in urine R^2-adjusted = 0.1244, n = 234.
[h] F in water R^2-Adjusted = 0.0928, n = 232; F in urine R^2-Adjusted = 0.0581, n = 236.
[i] F in water R^2-Adjusted = 0.2276, n = 230; F in urine R^2-Adjusted = 0.2274, n = 234.

urate crystals. The OPN models also showed a mean increase of 0.10 μg/mL in OPN per unit increase in water F concentration (p-value = .028) and a mean increase of 0.038 μg/mL in OPN per unit increase in urinary F concentration (p-value = .041); the models were adjusted by age, sex and the presence of amorphous urate crystals. The KIM-1 models were adjusted by the presence of amorphous urate crystals, the presence of mucoprotein, the atherogenic index and age; the models showed a positive and statistically significant association between KIM-1 excretion and F concentration in urine (β = 0.048 ng/mL, p-value = .008), and a not statistically significant association with F concentration in water (β = 0.045 ng/mL, p-value = .162). While TFF-3 models showed a significant association with F concentration in water (β = 2.88 ng/mL, p-value = .010) and a not statistically significant associatin with F concentration in urine (β = 1.14 ng/mL, p-value = .115); the models were adjusted by diabetes, age and sex. CLU models did not show any statistically significant association between the urinary excretion of the biomarker and the F concentration in water and urine (F in water model: β = 0.09 μg/mL, p-value = .118; F in urine model: β = 0.07 μg/mL, p-value = .100). Finally, the models for eGFR did not show a statistically significant association with water F concentration (β = 0.019 mL/min/1.73 m^2, p-value = .675) but estimated an average increase of 0.49 mL/min/1.73 m^2 per unit increase in

urine F concentration (p-value = .03); the models were adjusted by vascular disease, diabetes, serum cholesterol, alkaline phosphatases and nephrotoxic drug use. Similar results were obtained in models without urine normalization (Table S4). However, the models normalized by Creat values showed no significant associations with F concentration in water and urine and had lower R^2-adjusted values (Table S4). Sensitivity analysis showed regression coefficients in same direction and meaning when urinary F and urinary kidney biomarkers were normalized by specific gravity or when specific gravity was used as covariate in the models for urine adjustment (data not shown). No multicollinearity existed among the co-variates in the models. For all models co-linearity as variance inflation factor ranged from 1.01 to 1.19. In the initial analysis one potential outlier was identified in each model. Therefore, we used robust regression analysis that was less sensitive to outlier's impact. However, sensitivity analysis with and without outlier and using robust analysis were performed and regression coefficients are showed in Table S5. For most biomarkers, the regression coefficients showed same direction and meaning. However, in models with F concentration in urine a reduction in significance were observed for KIM-1 and eGFR without outlier, and non-statistical significance was observed for TFF-3 in robust regression. While in models with F concentration in water a slight significance reduction was observed without outlier.

Given that the population was also exposed to low As levels in drinking water, urinary As and F were highly correlated (Spearman's rho = 0.851, p-value < .0001, n = 236). In addition, we tested the possible interaction between urinary F and tAs levels to avoid multicollinearity and assess if the associations between kidney injury biomarkers and F concentration were modified by As exposure. The results showed no significant interactions (p-values > .1) in all kidney biomarker models (data not shown).

4. Discussion

The association between kidney damage and F exposure has been recognized in experimental studies. However, epidemiological data remain limited. The main purpose of this study was to assess the relationship between F exposure and the urinary excretion of early kidney injury biomarkers such as ALB, Cys-C, KIM-1 and OPN, and the potential interaction with As exposure in an adult Mexican population.

In the present study, we assessed F exposure using urinary F concentration and dental fluorosis as exposure biomarkers. The positive relationship between F concentration in water and urine as well as dental fluorosis suggest that drinking water may be the main source of chronic F exposure in the study population. Similar results were reported in other studies conducted in Chihuahua, Mexico, communities with natural F occurrence up to 11.8 mg/L in drinking water (González-Horta et al., 2015; Ruiz-Payan et al., 2005). Nevertheless, we do not discard possible F exposure contributions from other potential sources such as fluorinated salt, food background and pesticides (García-Pérez et al., 2013; Loyola-Rodríguez et al., 1998). The F concentration in water has been a useful marker to monitoring environmental F exposure. However, we consider the F concentration in urine can be a best marker to assess F exposure since it consider the individual variations in water consumption patterns and physiological conditions as well potential sources of F exposure, which is important consider, given that in Mexico high prevalence of dental fluorosis has been reported even in regions with low F concentration in drinking water (Aguilar-Díaz et al., 2017).

Urine is commonly used to assess F exposure and has been an important source of kidney function biomarkers. However, urine specimens have been shown to have high interindividual variations due to hydration status, and therefore, urine dilution adjustments are needed (Thomas et al., 2010). In the present study, we used three different methods to analyze urinary biomarker data: without adjustment, normalized by urinary Creat and normalized by urinary specific gravity. Urine Creat adjustment, which are commonly used, have some limitations that might lead to statistically biased results. Other urine dilution adjustment methods such as specific gravity have been proposed as alternatives (Weaver et al., 2016). However, there is no clear guidance in terms of unadjusted measurements or the best adjustment option for urinary biomarkers. In our study, we found similar results in unadjusted and urine specific gravity adjusted models. Therefore, we considered urinary specific gravity adjustment be more appropriate assessments of kidney injury biomarkers of F exposure.

In experimental studies, exposure to F has been associated with mitochondrial dysfunction, oxidative stress generation and apoptosis induction, especially in the PT, which is considered the section most susceptible to F toxicity (Barbier et al., 2010; Usuda et al., 1998). Nevertheless, few epidemiological studies have focused on assessing F nephrotoxicity, due in part to the lack of sensitive biomarkers to assess early kidney injury. Since 2008, the Food and Drug Administration (FDA) and the European Medicines Agency (EMEA) have approved the use of 7 urinary biomarkers (ALB, Cys-C, KIM-1, CLU, total protein, TFF-3 and β2-microglobulin) in preclinical studies (Dieterle et al., 2010); thus, these might be useful tools for assessing early kidney toxicity due to F exposure.

In normal conditions, low ALB levels can be filtered by the glomerulus and reabsorbed in the PT. Microalbuminuria (30–300 mg of ALB/g Creat) has been a well-established biomarker of CKD progression in diabetic nephropathy and hypertension (Basi et al., 2008). In our study, a low microalbuminuria prevalence was observed (3%) compared to that reported in the general US population (7.9%) (CDC, 2017). Nevertheless, a positive relationship between F exposure and urine ALB concentration was found, which is suggestive of a possible tubular dysfunction process that can precedes microalbuminuria. A similar relationship has been reported in populations environmentally exposed to uranium and low cadmium levels, which are well-established nephrotoxicants (Grau-Perez et al., 2017; Okaneku et al., 2015).

Cys-C is low molecular weight protein that is an inhibitor of cysteine proteases, produced by all nucleated cells, freely filtered in the glomeruli and reabsorbed and catabolized by PT cells (Filler et al., 2005). Urine Cys-C excretion is clearly associated with tubular dysfunction (Conti et al., 2006) and has been reported in diabetic nephropathy and acute and CKD (Kim et al., 2013; Park et al., 2013). Additionally, experimental studies have been shown a positive relationship between urine Cys-C levels and exposure to toxicants such as cadmium, cisplatin, gentamicin and paraquat (Prozialeck et al., 2016; Uchino et al., 2017; Wunnapuk et al., 2013). Despite the limited information about the use of urinary Cys-C to assess human chronic environmental kidney toxicity, we found a positive relationship between urinary Cys-C levels and F exposure, suggesting that urinary Cys-C might be a useful biomarker to assess kidney toxicity due to environmental exposure.

KIM-1 is a transmembrane glycoprotein up-regulated by PT cells after injury, with its soluble extracellular domain subsequently shed from cells into urine (Han et al., 2002). Acute KIM-1 expression was implicated in adaptive response, but its chronic expression has been related to maladaptive response and fibrosis development (Bonventre, 2014). Urinary KIM-1 levels are increased in patients with acute kidney injury, IgA nephropathy and diabetic nephropathy (Satirapoj et al., 2016; Shao et al., 2014; Xu et al., 2011). Additionally, urinary KIM-1 levels are one of the biomarkers most closely related to CKD progression and mortality (Waikar et al., 2016). Our results showed a positive relationship between urinary KIM-1 levels and F exposure. Similarly, increased urinary levels of KIM-1 have been reported in adults occupationally exposed to lead and environmentally exposed to cadmium (Ruangyuttikarn et al., 2013; Zhou et al., 2016) and in Mexican children environmentally exposed to chromium and As (Cárdenas-González et al., 2016), which indicates a possible early kidney injury related to toxicant exposure.

OPN [bone sialoprotein I (SBP-I), early T-lymphocyte activation (ETA-1), uropontin-secreted phosphoprotein 1 (SSP 1)] is a

glycoprotein expressed by several type cells such as osteoblasts, endothelial cells, macrophages, T-cells and kidney cells, where it is involved in bone biomineralization, tissue remodeling and inflammation (Kahles et al., 2014). In the normal adult kidney, OPN is primarily expressed by the thin ascending limb of the loops of Henle, but in cases of interstitial fibrosis and influxes of interstitial macrophages, it is expressed in all tubule segments, and it is also involved in several pathophysiological processes (Xie et al., 2001). We found a relationship between urinary OPN excretion and F exposure. Although limited epidemiological information about toxic exposure and urine OPN is available, increased urine OPN levels have been observed in patients with acute kidney disease, lupus nephropathy and CKD (Feldreich et al., 2017; Kitagori et al., 2016; Vaidya et al., 2008).

TFF-3 is a small peptide hormone secreted by mucus-producing and other epithelial cells. In the kidney, TFF-3 is mainly produced by proximal tubule and collecting duct cells, and increased urine excretion has been reported in CKD patients (Astor et al., 2011; O'Seaghdha et al., 2013). In addition, CLU is a glycosylated protein that has been suggested to participate in anti-apoptotic processes and the clearance of cellular debris (Cunin et al., 2016). Both biomarkers have been shown to be involved in the kidney repair process, but their biological importance in the kidney is not fully understood (Guo et al., 2016; Hoffmann, 2005). Despite their association with kidney injury in animal models, no extensive clinical information has been reported (Dvergsten et al., 1994; Hidaka et al., 2002; Yu et al., 2010). In our study, non-significant relationships between urine TFF-3 and CLU levels and F exposure were found. We speculate that this might be due in part to their non-specificity in detecting proximal tubule damage, which is considered the main target of F toxicity, and to their association with other non-kidney diseases and aging (Debata et al., 2007; Stejskal and Fiala, 2006). Nevertheless, more studies are necessary to assess their use as biomarkers in human nephrotoxicity.

In relation to the eGFR, one of the most accepted biomarker in the evaluation of kidney function, used to define the stage of CDK (KDIGO, 2013). In the present study, a positive relationship between F exposure and the eGFR was observed. Similarly, increased eGFR have been reported in US populations environmental exposed to cadmium and lead (Buser et al., 2016) and in lead workers (Weaver et al., 2011). The hyperfiltration process, characterized by increased eGFR followed by subsequent declines, is a potential explanation for this unexpected result that has been reported in patients with diabetes, hypertension and lead exposure (Khalil-Manesh et al., 1992; Palatini, 2012). However, we do not discard a possible reverse causality effect (Weaver et al., 2016), which could be due to low F excretion due to kidney filtration impairments in the study population.

In the present study, we show data from a panel of 4 early kidney injury biomarkers and their positive relationships with F exposure. Similar outcomes were reported in a previous study with sub-chronically exposed animals to relevant F doses to those of human exposure (Cárdenas-González et al., 2013). Thus, these results suggest a possible association between F exposure and tubular injury, which has been associated with CKD development (Hodgkins and Schnaper, 2012).

F and As co-occurrence in groundwater sources is common in many regions worldwide. Experimental and epidemiological studies have linked As exposure to kidney impairments (Robles-Osorio et al., 2015). Nevertheless, limited information is available in terms of As-F co-exposure nephrotoxic effects. In our study, we did not find an interaction effect of urinary As concentration with any kidney biomarker model. We speculate that this might be due to the relative low As exposure levels compared to other epidemiological studies (Zheng et al., 2014). However, additional studies, especially those performed in the highest As exposure regions, are necessary to assess F-As co-exposure nephrotoxicity effects.

Our findings advance epidemiological information about potential F nephrotoxicity in several ways. The main strength of our study was the assessment of early kidney injury biomarkers in a F exposed adult population considering adjusted variables and As exposure, which has been never performed before. However, some limitations should be considered when interpreting our results. Due to our cross-sectional study design, no causation can be established. Additionally, higher proportion of females than males and no other potential confounding factors such as nutritional status, physical activity or other nephrotoxicants exposure were analyzed.

In conclusion, the present study has shown that the urinary excretion of 4 early kidney injury biomarkers (ALB, Cys-C, KIM-1 and OPN) is related to environmental F exposure in an adult population, without an As interaction effect. Our results suggest a possible tubular dysfunction from F exposure that might increase susceptibility to the future development of CKD. Further studies are needed to clarify F contributions to CKD development/progression, F nephrotoxic effects in susceptible populations, and F co-exposure effects in those exposed to high levels of As.

Conflict of interest

The authors declare that there is no conflict of interest.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.taap.2018.05.027.

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