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ORIGINAL ARTICLE

Urinary fibrogenic cytokines ET-1 and TGF-β1 are associated with urinary angiotensinogen levels in obese children

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Abstract

Background Fibrogenic cytokines are recognized as putative drivers of disease activity and histopathological deterioration in various kidney diseases. We compared urinary transforming growth factor β1 (U-TGF-β1) and endothelin 1 (U-ET-1) levels across body mass index classes and assessed their association with the level of urinary angiotensinogen (U-AGT), a biomarker of intrarenal renin–angiotensin–aldosterone system (RAAS).

Methods The was a cross-sectional evaluation of 302 children aged 8–9 years. Ambulatory blood pressure (BP), insulin resistance (HOMA-IR), aldosterone level and renal function were evaluated. U-ET-1, U-TGF-β1 and U-AGT levels were determined by immunoenzymatic methods.

Results Obese children presented with the lowest levels of U-ET-1 and U-TGF-β1, but the difference was only significant for U-ET-1. In obese children, the median levels of both U-ET-1 and U-TGF-β1 tended to increase across tertiles (T1–T3) of U-AGT (U-ET-1: T1, 19.9 (14.2–26.3); T2, 32.5 (23.3–141.6); T3, 24.8 (18.7–51.5) ng/g creatinine, $p=0.007$; U-TGF-β1: T1, 2.2 (1.8–4.0); T2, 4.3 (2.7–11.7); T3, 4.9 (3.8–10.1) ng/g creatinine, $p=0.004$]. In multivariate models, in the obese group, U-ET-1 was associated with HOMA-IR and aldosterone and U-AGT levels, and U-TGF-β1 was associated with U-AGT levels and 24 h-systolic BP.

Conclusions Whereas the initial hypothesis of higher levels of urinary fibrogenic cytokines in obese children was not confirmed in our study, both TGF-β1 and U-ET-1 levels were

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associated with U-AGT level, which likely reflects an early interplay between tissue remodeling and RAAS in obesity-related kidney injury.

Keywords Childhood obesity · Kidney injury · Endothelin-1 · Transforming growth factor-β1 · Angiotensinogen · Urinary biomarkers

Introduction

The prevalence of obesity has been steadily increasing worldwide over the past few decades [1, 2]. Obesity has been identified as a strong, independent and potentially modifiable risk factor for the development and progression of kidney disease, both in adults and in children [3, 4]. Several mechanisms are thought to contribute to this association [5]. Obesity-related kidney damage is initially characterized by hyperfiltration [6] that later potentiates progressive renal damage, with increased loss of proteins and cellular remodeling and tubulo-interstitial fibrosis [7]. In fact, tissue remodeling and fibrosis represent a common pathway in all kidney diseases and are known to be associated with the local release of several biologically active factors [8], such as endothelin-1 (ET-1) and transforming growth factor beta 1 (TGF-β1).

ET-1 is a potent vasoconstrictor that is expressed in several kidney cell types and plays a role in the interplay between interstitial fibrosis and glomerulosclerosis [9, 10]. TGF-β1 is ubiquitously expressed and is involved in extracellular matrix remodeling, renal fibrogenesis and nephron loss, thus playing an important role in the progression of chronic kidney disease (CKD) [11]. The urinary excretion of both ET-1 (U-ET-1) and TGF-β1 (U-TGF-β1) reflects their intrarenal production, suggesting that both might be used as markers of tissue damage severity [12, 13]. In children, the levels of both U-ET-1 and U-TGF-β1 increase in a wide spectrum of kidney conditions, such as obstructive uropathies [14], reflux nephropathy [15] and CKD of different etiologies [16], among others. In addition, the level of U-TGF-β1 correlates with the degree of renal interstitial fibrosis [17].

Urinary cytokine levels are also increased in children with congenital single kidneys, even in early phases of hyperfiltration when proteinuria and renal insufficiency are still absent [18]. Since kidney diseases with reduced renal mass are believed to mimic what happens in obesity, representing a clinical translation of experimental hyperfiltration models [7], we hypothesized that levels of urinary cytokines might also be increased in overweight and obese children. This increase would then reflect some initial degree of inflammation and renal hemodynamic changes.

ET-1 has been proposed as a regulator of the renal renin–angiotensin–aldosterone system (RAAS) in the setting of obesity [19, 20]. Renin and angiotensin II may also directly stimulate the TGF-β1 pathway in the kidney [21], independently of renal hemodynamics [22]. Thus, levels of U-ET-1 and U-TGF-β1 might also indicate overactivation of the renal RAAS. This link between renal production of ET-1 and TGF-β1 and the intrarenal RAAS has not been described yet in the setting of childhood obesity. Therefore, our aim was to compare the levels of U-TGF-β1 and U-ET-1 in normal weight, overweight and obese children and to assess the association of these urinary cytokines with urinary angiotensinogen (U-AGT), a biomarker of the intrarenal RAAS [23].

Methods

Study design and sample

We conducted a cross-sectional study of children aged 8–9 years who had been followed since birth in a cohort study (Generation XXI, Porto-Portugal) [24]. Subjects from the original cohort were eligible for recruitment to the present study protocol (ObiKid project) if anthropometric data and a blood sample had been obtained at the 7-year evaluation (n= 4,590). Our aim was to include a minimum sample of 300 children for the Obikid project's main objective. Assuming a 35 % dropout rate due to refusal to participate, exclusion criteria or incomplete information, 463 children were preselected to be consecutively screened according to the date of their 7-year evaluation. Of these 463 children, 16 could not be contacted, 32 refused to participate, 23 were unable to schedule the study visits during the recruitment period and 68 met the exclusion criteria [4 with chronic diseases (genetic, renal or metabolic), 1 with chronic usage of medication (affecting blood pressure, glucose or lipid metabolism), 51 living (too) far from the study site and 12 twins]. The remaining 324 participants were enrolled in the study, but 22 were excluded between August 2013 and August 2014due to the lack of adequate urine samples for the determination of cytokine levels, leaving 302 children for the final analysis.

Data collection and definition of variables

Anthropometric measurements were obtained and a general physical examination was performed according to standard procedures and as previously reported [25]. Waist circumference was indexed to height [waist-to-height ratio). The body mass index (BMI) was calculated, and BMI-for-age values were classified according to the World Health Organization reference data for BMI z-scores into the following categories: normal weight [≤+1 standard deviation (SD)], overweight (>1SD and ≤+2SD) and obesity (>2SD) [26]. Body fat percentage was assessed by foot-to-foot bioelectrical impedance analysis (TBF-300; Tanita Corp., Tokyo, Japan).

Ambulatory BP monitoring (ABPM) for 24 h was performed in all children with a portable non-invasive oscillometric BP recorder (Spacelabs Healthcare® model 90207; OSI Systems, Hawthorne, CA), in the non-dominant arm with an appropriate cuff size. A minimum monitoring duration of 24 h with gaps of <2 h was required for the data set; 5 sets of readings were excluded from the ABPM analysis due to insufficient readings. Office BP was evaluated a validated oscillometric sphygmomanometer (model Elite 92125; Medel, Parma, Italy), with an adequately sized cuff, in the right arm, by a trained non-physician interviewer. Three BP measurements were taken, with a 5-min interval between measurements, and the second and third measurements were averaged for further analysis. Sustained hypertension [27] was defined as an average systolic BP (SBP) and/or diastolic BP (DBP) measurement of ≥95th percentile, either during the day or night on ABPM, according to the reference values [27], a SBP or DPB load of ≥25 %, during either the day or night, and an office SBP and/or DBP of ≥95th percentile, according to the American Academy of Pediatrics criteria [28]. When office BP values were <95th percentile, but the remaining criteria were verified, the children were classified as presenting masked hypertension [27]. The absence of dipping was considered to be a fall in the mean arterial pressure (MAP) during nighttime of <10 % of the corresponding daytime MAP.

Laboratory procedures

For all participants, a random spot urine sample was collected in the morning and a venous blood sample was collected after an overnight fast of at least 8 h.

The serum creatinine assay was based on the compensated Jaffé methods and traceable to an isotope dilution mass spectrometry method (Olympus AU 5400 automated analyzer; Beckman Coulter, Brea, CA). Serum cystatin C was assayed using a particle-enhanced immunonephelometric assay (N latex Cystatin C; Siemens AG, Erlangen, Germany). The Zappitelli combined formula was used to estimate glomerular filtration rate (eGFR) [29]. Aldosterone concentration in serum samples was measured using the Liaison Aldosterone kit® (DiaSorin Inc., Saluggia, Italy). Insulin resistance was determined using the homeostasis model assessment index (HOMA-IR) [30].

Urinary creatinine and albumin were determined by a standard automated clinical chemistry analyzer (Olympus AU 5400 automated analyzer; Beckman-Coulter). Urine and blood samples were frozen immediately after collection and stored in aliquots at −80 °C with no additives until further processing for the U-ET-1, U-TGF-β1, U-AGT and plasma (P)-AGT measurements; the samples were not subjected to freeze–thaw cycles that could interfere with the results. These measurements were made by the Department of Pharmacology and Therapeutics of the Faculty of Medicine of the University of Porto using immunoenzymatic methods with commercial enzyme-linked immunosorbent assay (ELISA) kits, according to the protocol provided by the manufacturer with slight modifications for U-ET-1 and U-TGF-β1 quantifications (U-ET-1: Biomedica, Medizinprodukte GmbH & Co KG, Austria; U-TGF-β1: R&D Systems, Minneapolis, MN; AGT and U-AGT/P-AGT: Total Angiotensinogen Assay kit, Immuno-Biological Laboratories, Tokyo, Japan). Briefly, for quantification of U-ET-1, absorbance values from urine samples were interpolated in a calibration curve in the range of 0–2 fmol/ml. For quantification of U-TGF-β1, an ELAST® ELISA Amplification System (PerkinElmer, Waltham, MA) was used to amplify the signal generated by the enzyme horseradish peroxidase (HRP). Specifically, following incubation with HRP (and washing) and before the addition of the chromogenic substrate, the ELAST® procedure was performed using a biotinyl-tyramide solution (1:10) and streptavidin-HRP solution (1:500). The limit of detection of the U-ET-1 and U-TGF-β1 kits was 0.04 fmol/mL and 3.54 pg/mL, respectively. Samples with levels under this detection limit were arbitrarily assigned the value 0.02 fmol/mL for U-ET-1 and 1.75 pg/mL for U-TGF-β1. All values obtained from spot urine samples were expressed as normalized for urinary creatinine.

Statistical analysis

Standard statistical analysis was performed using IBM SPSS Statistics for Windows, Version 20.0 (IBM Corp., Armonk, NY). Data are presented as the mean and SD or, if skewed, as the median and interquartile range (IQR). U-ET-1 and U-TGF-β1 had an asymmetric distribution and were logarithmized (base 10) before the linear regression analyses to obtain a normal distribution. Linear regression models were used to identify variables independently associated with U-ET-1 and U-TGF-β1, using log-transformed values of both urinary cytokines. A final multivariate model was fitted for each cytokine, including all of the variables that in the univariate analysis were associated with the cytokine levels at *p*-values of <0.25, as well as sex and age (in months). Only 24-h SBP was included in all models, since the inclusion of both SBP and DBP would introduce collinearity and over-adjustment. All linear regression models were stratified by BMI *z*-score classes because there was a significant interaction between BMI classes and U-AGT levels.

Results

A total of 302 children with a mean (SD) age of 8.8 (0.2) years were included in our cross-sectional evaluation. General characteristics, ABPM values and biochemical parameters are

shown in Table 1, according to classes of BMI (normal weight, n=159; overweight, n=84; obese, n=59). Overweight and obese children presented significantly higher values of 24-h SBP, insulin resistance, high-sensitivity C-reactive protein (hsCRP), uric acid, plasma creatinine and cystatin C, and lower eGFR. No differences were found across groups for the median blood values of interleukin -6 (IL-6), aldosterone and P-AGT, for urinary albumin values or for U-AGT excretion.

Figure 1 depicts the distribution of U-ET-1 and U-TGF-β1 according to BMI classes. For U-ET-1, significant differences were found between groups, with the highest levels in overweight children and the lowest in obese children [normal weight vs. overweight vs. obesity groups: 27.1 (IQR 19.1–27.1) vs. 33.5 (22.0–52.6) vs. 24.4 (18.3–36.7) fmol/g creatinine, respectively, p=0.032] The median levels of U-TGF-β1 were also lowest in obese children [normal weight vs. overweight vs. obesity groups: 5.4 (IQR 2.7–10.0) vs. 5.1 (3.3–9.4) vs. 3.8 (2.2–7.0) ng/g creatinine, respectively; p=0.060)]

In the univariate analysis, significant associations were found between U-ET-1 levels and 24-h SBP and DBP, HOMA-IR and aldosterone in the normal weight group and between U-ET-1 levels and HOMA-IR and U-AGT in the obese group (Table 2). For U-TGF-β1, the only significant associations were found with U-AGT in both the overweight and obese groups and with eGFR levels in only the overweight group (Table 2). The association of U-AGT and each

Table 1 General anthropometric and clinical characteristics and laboratory parameters of the study subjects according to body mass index z-score classes

General characteristics	World Health Organization BMI z-score classification[a]			p[b]
	Normal weight (n=159)	Overweight (n=84)	Obese (n=59)	
anthropometric and clinical characteristics				
Age (months)	105.1±2.9	105.3±2.7	105.6±2.9	0.519
Male sex	82 (52 %)	42 (50 %)	38 (64 %)	0.176
BMI z-score	−0.02±0.73	1.55±0.30	2.67±0.48	<0.001
WHtR (cm/m)	44.7±2.5	50.1±3.2	56.8±4.4	<0.001
% body fat mass	10.7±7.2	19.9±8.0	28.3±9.3	<0.001
24-h Ambulatory blood pressure				
24-h SBP (mmHg)	111.1±6.4	113.6±7.1	114.3±8.7	0.004
24-h DBP (mmHg)	66.8±4.1	67.2±5.3	66.6±6.0	0.701
Sustained hypertension/masked hypertension[c]	1 (0.6 %)/8 (5.0 %)	3 (3.6 %)/5 (6.0 %)	3 (5.1 %)/6 (10.2 %)	–
Absence of dipping	34 (22 %)	28 (33 %)	17 (29 %)	0.143
Blood analytical parameters				
HOMA-IR	1.12 (0.84–1.36)	1.34 (1.06–1.84)	1.71 (1.30–2.63)	<0.001
hsCRP (mg/L)	0.0 (0.0–0.4)	0.5 (0.2–1.2)	0.7 (0.4–1.7)	<0.001
IL-6 (pg/mL)	0.75 (0.75–2.43)	1.60 (0.75–3.00)	1.97 (0.75–2.99)	0.096
Uric acid (mg/dL)	3.48±0.66	3.72±0.71	4.02±0.75	<0.001
Creatinine (mg/dL)	0.43±0.06	0.45±0.06	0.45±0.06	0.005
Cystatin-C (mg/L)	0.64±0.07	0.67±0.08	0.69±0.06	<0.001
eGFR (mL/min/1.73 m^2)	138.3±15.7	132.5±16.4	132.0±13.2	0.003
Aldosterone (ng/dL)	9.2 (7.0–13.2)	12.1 (7.3–16.5)	9.7 (8.4–12.9)	0.108
P-AGT (μg/mL)	36.4 (32.4–47.3)	37.0 (32.5–44.2)	38.8 (34.1–44.1)	0.665
Urinary analytical parameters				
U-Albumin (mg/g creatinine)	3.8 (2.6–7.2)	3.7 (2.2–7.4)	3.9 (2.3–6.2)	0.874
U-AGT (μg/g creatinine)	5.5 (3.8–7.7)	5.2 (3.2–8.6)	4.8 (2.8–7.8)	0.418

The values are presented as the mean±standard deviation (SD) or as the median with the interquartile range (IQR) in parenthesis, for continuous variables with and without normal distribution, respectively. Categorical variables are shown as a number (n) with the percentage in parenthesis

BMI, body mass index; WHtR, waist-to-height ratio; SBP, systolic blood pressure; DBP, diastolic blood pressure; HOMA-IR, homeostasis model assessment of insulin resistance; hsCRP, high sensitivity C-reactive protein; IL-6, interleukin-6; eGFR, estimated glomerular filtration rate by combined Zappitelli formula; P-AGT, plasma angiotensinogen; U-AGT, urinary angiotensinogen

[a] The BMI z-score classification is according to the World Health Organization criteria [26]

[b] p values were calculated by the one-way analysis of variance (ANOVA) test or by the Kruskal–Wallis test, respectively

[c] Sustained hypertension and masked hypertension were defined according to the revised American Heart Association criteria for ABPM interpretation [27]

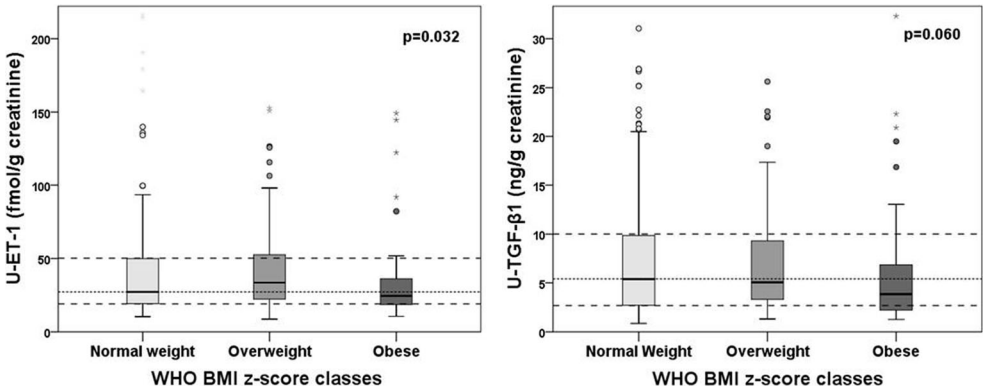

Fig. 1 Distribution of urinary endothelin 1 (*U-ET-1*) and transforming growth factor beta 1 (*TGF-β1*) according to classes of body mass index (*BMI*; Normal weight, Overweight, Obesity). The normal weight, overweight and obese group classification is according to the World Health Organization (*WHO*) classification for BMI *z*-score values [26]. The U-ET-1 and U-TGF-β1 data are expressed the median (*horizontal line in box*) and interquartile range (IQR: 25th–75th percentiles; *upper and lower limits of box*). *p* values were calculated using the Kruskal–Wallis test

of the urinary cytokines was found to be significant in obese children but not in normal weight children (*p* for interaction= 0.047 and 0.049 for U-ET-1 and U-TGF-β1, respectively). The relationship between urinary cytokines and U-AGT, stratified by BMI classes, is shown in Fig. 2. In obese children, the levels of both U-ET-1 and U-TGF- β1 increased with rising U-AGT [U-ET-1: 1st tertile (T1), 19.9 (IQR 14.2–26.3); 2nd tertile (T2), 32.5 (23.3–141.6); 3rd tertile (T3), 24.8 (18.7–51.5) ng/g creatinine, p=0.007; U-TGF- β1: T1, 2.2 (IQR 1.8–4.0); T2, 4.3 (2.7–1.7); T3, 4.9 (3.8–10.1) ng/g creatinine, p=0.004].

In the multivariate regression models, in the normal weight group, the log of U-ET-1 increased significantly with 24-h SBP [mean increase 0.010 (IQR 0.002–0.019) fmol/g creatinine per mmHg; p=0.025] and with HOMA-IR [mean increase 0.109 (IQR 0.010–0.208) fmol/g creatinine per unit; p=0.031)]. In the obese children, U-ET-1 was independently associated with HOMA-IR [+0.145 (IQR 0.044–0.246) fmol/g creatinine per unit; p=0.006], aldosterone [−0.038 (IQR −0.060 to −0.016) fmol/g creatinine per ng/dL; p=0.001)] and U-AGT [+0.155 (IQR 0.033–0.277) fmol/g creatinine per tertile of U-AGT; p=0.014]. In the U-TGF-β1 model, no significant adjusted associations were found for normal weight or overweight children, and in obese children the log of U-TGF-β1 was significantly associated only with U-AGT, increasing by only 0.162 (IQR 0.046 to 0.278) fmol/g creatinine per tertile of U-AGT (p=0.007), and 24-h SBP, increasing by 0.012 (IQR 0.001–0.023) fmol/g creatinine per mmHg (p=0.029) (Table 3).

Discussion

Our study highlights the possibility that ET-1, TGF-β1 and renal AGT play interacting roles in obesity-related kidney injury which have not previously been addressed in healthy young children. Contrary to our initial hypothesis, the prepubertal obese children, otherwise healthy, in our study did not have higher levels of the fibrogenic cytokines ET-1 and TGF-β1 in their urine, but rather they had the lowest levels. However, in these obese children we did find significant associations between the levels of U-ET-1 and U-TGF-β1 and the urinary excretion of AGT, a marker of intrarenal RAAS activity.

Our initial hypothesis that urinary excretion of fibrogenic cytokines might be increased in obese children was based on several indirect findings. Urinary excretion of fibrogenic cytokines has been found to be higher in several kidney diseases [31–34] and, more specifically, in children with congenital single kidney disease [35], a clinical setting in which hyperfiltration occurs, similar to what happens in obesity. Moreover, a study in adults with essential hypertension found that those with higher BMI presented higher U-TGF-β1 levels [36]. One possible explanation for our findings is that the children of our study are at an early phase of obesity-related kidney injury, when a still unknown mechanism of regulation might be operating in an attempt to protect the kidney. Interestingly, U-ET-1 has been suggested as a useful marker of renal inflammation in the early stages of inflammatory renal disease—before renal function is affected [31]. Further explorations of the levels of urinary fibrogenic cytokines associated with obesity and of different pediatric ages might help to clarify the interpretation of our results. Given the lack of previous studies, we advise that our findings be interpreted cautiously.

A particularly important finding of our study is the positive association of both U-ET-1 and U-TGF-β1 with U-AGT in obese children. It is well established that in obesity there is a chronic overactivation of the fat RAAS [32] and that the increased release of adipose tissue-derived RAAS components into circulation affects hemodynamic homeostasis and the intrarenal RAAS itself [33]. U-AGT is a non-invasive marker of intrarenal RAAS activity [37]. The positive association between urinary levels of fibrogenic cytokines, which has

Table 2 Univariate linear regression models for urinary urinary endothelin 1 and urinary transforming growth factor beta 1 in normal weight, overweight and obese children[a]

Univariate linear regression models	Normal weight		Overweight		Obese	
	Crude β (95 % CI)	*p*	Crude β (95 % CI)	*p*	Crude β (95 % CI)	*p*
Log U-ET-1 (fmol/g creatinine)						
Age (monthw)	0.021 (0.002–0.040)	0.027	−0.008 (−0.037 to 0.021)	0.589	0.021 (−0.017 to 0.058)	0.284
24-h SBP (mmHg)	0.014 (0.006–0.023)	0.001	−0.009 (−0.020 to 0.002)	0.115	−0.002 (−0.015 to 0.010)	0.717
24-h DBP (mmHg)	0.015 (0.002–0.028)	0.029	0.003 (−0.012 to 0.019)	0.656	−0.003 (−0.021 to 0.015)	0.772
HOMA-IR (units)	0.155 (0.056–0.255)	0.002	0.033 (−0.087 to 0.153)	0.588	0.109 (0.017–0.200)	0.021
hsCRP (mg/L)	−0.015 (−0.059 to 0.029)	0.503	−0.001 (−0.028 to 0.026)	0.931	−0.014 (−0.067 to 0.039)	0.605
IL-6 (pg/mL)	0.004 (−0.016 to 0.023)	0.690	0.013 (−0.024 to 0.051)	0.483	0.000 (−0.061 to 0.061)	0.991
Uric acid (mg/dL)	0.011 (−0.072 to 0.095)	0.792	−0.021 (−0.133 to 0.090)	0.707	0.063 (−0.083 to 0.208)	0.393
eGFR (mL/min/1.73 m^2)	0.003 (0.000–0.007)	0.075	−0.003 (−0.008 to 0.002)	0.194	0.008 (0.000–0.016)	0.062
Aldosterone (ng/dL)	−0.014 (−0.024 to −0.003)	0.011	−0.004 (−0.018 to 0.010)	0.540	−0.023 (−0.047 to 0.000)	0.052
P-AGT (per tertile)	−0.026 (−0.093 to 0.041)	0.439	0.037 (−0.062 to 0.135)	0.458	0.033 (−0.108 to 0.174)	0.637
U-Albumin (mg/g creatinine)	0.001 (−0.001–0.002)	0.389	0.003 (−0.001 to 0.008)	0.155	0.000 (−0.002 to 0.001)	0.902
U-AGT (per tertile)	0.009 (−0.060 to 0.079)	0.793	−0.050 (−0.150 to 0.049)	0.316	0.131 (0.007–0.254)	0.038
Log U-TGF-β1 (ng/g creatinine)	0.121 (−0.021 to 0.263)	0.093	−0.036 (−0.249 to 0.178)	0.742	0.202 (−0.089 to 0.492)	0.170
Log U-TGF-β1 (ng/g creatinine)						
Age (months)	−0.034 (−0.054 to −0.014)	0.001	−0.024 (−0.054 to 0.005)	0.104	−0.026 (−0.059 to 0.008)	0.134
24-h SBP (mmHg)	−0.001 (−0.011 to 0.008)	0.803	0.004 (−0.007 to 0.016)	0.443	0.009 (−0.002 to 0.020)	0.119
24-h DBP (mmHg)	0.000 (−0.015 to 0.015)	0.989	0.005 (−0.010 to 0.021)	0.496	0.013 (−0.003 to 0.029)	0.114
HOMA-IR (units)	−0.044 (−0.155 to 0.066)	0.428	0.022 (−0.102 to 0.145)	0.729	−0.043 (−0.129 to 0.043)	0.321
hsCRP (mg/L)	0.007 (−0.042 to 0.055)	0.791	0.010 (−0.018 to 0.037)	0.487	−0.016 (−0.064 to 0.032)	0.510
IL-6 (pg/mL)	−0.009 (−0.032 to 0.013)	0.408	0.008 (−0.031 to 0.046)	0.700	−0.016 (−0.071 to 0.038)	0.556
Uric acid (mg/dL)	−0.090 (−0.181 to 0.001)	0.053	−0.091 (−0.204 to 0.021)	0.110	−0.090 (−0.219 to 0.040)	0.171
eGFR (mL/min/1.73 m^2)	0.002 (−0.002 to 0.006)	0.233	0.005 (0.000–0.010)	0.045	0.000 (−0.007 to 0.008)	0.894
Aldosterone (ng/dL)	−0.006 (−0.018 to 0.006)	0.314	−0.008 (−0.022 to 0.007)	0.288	−0.011 (−0.032 to 0.011)	0.321
P-AGT (per tertile)	−0.025 (−0.098 to 0.048)	0.503	0.092 (−0.008 to 0.191)	0.070	−0.012 (−0.139 to 0.115)	0.848
U-Albumin (mg/g creatinine)	0.000 (−0.001 to 0.002)	0.794	0.003 (−0.001 to 0.008)	0.178	0.001 (−0.001 to 0.002)	0.437
U-AGT (per tertile)	0.064 (−0.013 to 0.141)	0.100	0.103 (0.003 to 0.203)	0.043	0.172 (0.065 to 0.280)	0.002
Log U-ET-1 (ng/g creatinine)	0.148 (−0.025 to 0.321)	0.093	−0.037 (−0.263 to 0.188)	0.742	0.163 (−0.072 to 0.397)	0.170

U-ET-1, Urinary endothelin-1; TGF-β1, transforming growth factor beta 1; SBP, systolic blood pressure; DBP, diastolic blood pressure; eGFR, estimated glomerular filtration rate; hCRP, high sensitivity C-reactive protein

[a] The values presented are adjusted linear regression coefficients (β) and 95 % confidence intervals (CI), estimated by univariate linear regression models with urinary ET-1 (logarithm base 10) or with urinary TGF-β1 (logarithm base 10) as the dependent variables. The results are presented separately by BMI *z*-score classes because the effect of U-AGT on each of the urinary cytokines was found to be significant in obese children but not in normal weight children (*p* for interaction=0.047 and 0.049 for urinary ET-1 and TGF-β1, respectively)

been shown to reflect their intrarenal production [30, 36], and U-AGT potentially underlies a mechanism through which the RAAS and cytokines regulate each other in obesity. Also, in an experimental model, obesity was found to be associated with increased angiotensin-converting enzyme activity, specifically in the kidney, in an ET_A receptor-dependent manner [38]. Should this renal RAAS–cytokine interaction be sustained, it would support a deleterious pathway in which normal cytokine-mediated tissue repair in response to acute injury would give place to progressive fibrosis that ultimately causes kidney impairment. Thus, our findings fit into the hypothesis that ET-1 acts as a regulator of the local renal RAAS in the setting of obesity [38, 39] and that RAAS components directly stimulate the TGF-β1 pathway in the kidney [40]. Taken together, our results suggest that even in the early phases of kidney injury inflicted by excessive adipose mass, the association between urinary cytokines and intrarenal RAAS may already exist. Thus, even though the obese children in our study presented the lowest levels of urinary cytokines, which, as previously stated, might result from a still unknown mechanism of regulation operating in early phases of obesity and obesity-related kidney injury, the fact that an association with U-AGT existed exclusively in this group

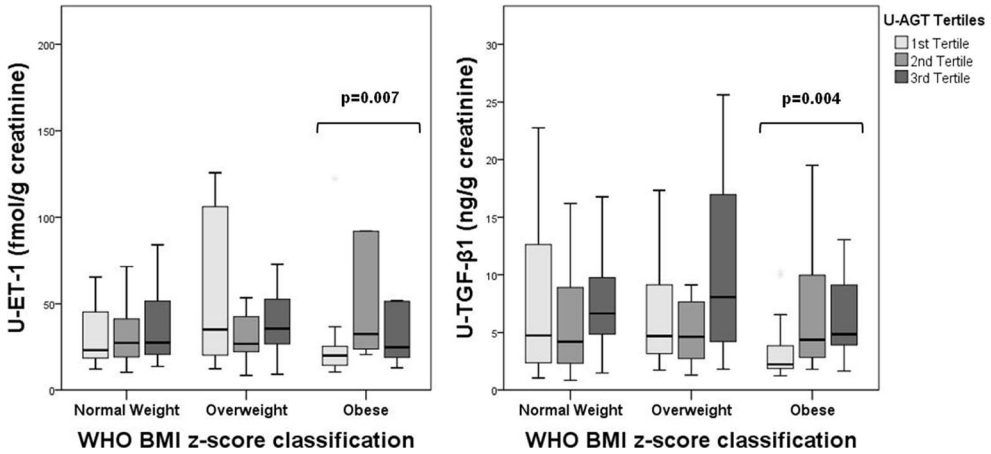

Fig. 2 Distribution of U-ET-1 and U-TGF-β1 by tertiles of urinary angiotensinogen (*U-AGT*) separately in the normal weight, overweight and obesity groups, as classified according to the WHO classification for body mass index *z*-score values [26]. The tertiles of urinary AGT (T1, ≤4.08; T2, 4.08–6.69; T3, >6.69) were based on data from all enrolled participants. The urinary ET-1 and TGF-β1 data are expressed as medians (horizontal line in box) and IQR (25th–75th percentile; *upper and lower limits of box*). *p* values were calculated using the Kruskal–Wallis test. *Significant at $p<0.01$

probably represents a point of interaction between these systems that arises in the setting of obesity and which requires further exploration in future studies. As previously stated, these findings are novel and have not been previously tested in human trials with pediatric age subjects, reinforcing the need for a prudent interpretation of our findings and confirmation in further studies.

The negative association which we observed between U-ET-1 and serum aldosterone levels is difficult to interpret. It has been proposed that aldosterone itself may also play a role

Table 3 Multivariate linear regression models for urinary urinary endothelin 1 and urinary transforming growth factor beta 1 in normal weight, overweight and obese children

Multivariate linear regression models	Normal weight		Overweight		Obese	
	Adjusted β (95 % CI)	*p*	Adjusted β (95 % CI)	*p*	Adjusted β (95 % CI)	*p*
Log U-ET-1 (fmol/g creatinine)						
24-h SBP (mmHg)	0.010 (0.001–0.019)	0.025	−0.008 (−0.020 to 0.003)	0.163	−0.004 (−0.015 to 0.008)	0.500
HOMA-IR (unit)	0.109 (0.010–0.208)	0.031	0.025 (−0.105 to 0.154)	0.706	0.145 (0.044–0.246)	0.006
eGFR (mL/min/1.73 m^2)	0.002 (−0.001 to 0.006)	0.179	−0.004 (−0.009 to 0.001)	0.145	0.005 (−0.002 to 0.012)	0.145
Aldosterone (ng/dL)	−0.008 (−0.019 to 0.003)	0.135	−0.010 (−0.025 to 0.005)	0.201	−0.038 (−0.060 to −0.016)	0.001
U-Albumin (mg/g creatinine)	0.001 (−0.001 to 0.002)	0.400	0.004 (−0.001 to 0.009)	0.119	−0.008 (−0.026 to 0.011)	0.409
U-AGT (per tertile)	0.007 (−0.063 to 0.077)	0.846	−0.080 (−0.192 to 0.032)	0.161	0.155 (0.033–0.277)	0.014
Log U-TGF-β1 (ng/g creatinine)	0.145 (−0.007 to 0.296)	0.061	−0.018 (−0.254 to 0.217)	0.877	0.083 (−0.204 to 0.370)	0.564
Log U-TGF-β1 (ng/g creatinine)						
24-h SBP (mmHg)	0.002 (−0.008 to 0.012)	0.697	0.007 (−0.004 to 0.019)	0.216	0.012 (0.001–0.023)	0.028
Uric acid (mg/dL)	−0.074 (−0.165 to 0.017)	0.111	−0.056 (−0.177 to 0.066)	0.364	−0.085 (−0.216 to 0.047)	0.200
eGFR (mL/min/1.73 m^2)	0.001 (−0.003 to 0.005)	0.547	0.003 (−0.002 to 0.008)	0.270	−0.001 (−0.008 to 0.007)	0.853
P-AGT (per tertile)	−0.010 (−0.083 to 0.063)	0.783	0.071 (−0.030 to 0.172)	0.165	−0.048 (−0.178 to 0.083)	0.466
U-Albumin (mg/g creatinine)	0.000 (−0.002 to 0.001)	0.908	0.001 (−0.005 to 0.005)	0.910	−0.006 (−0.025 to 0.012)	0.488
U-AGT (per tertile)	0.030 (−0.048 to 0.108)	0.452	0.108 (0.001 to 0.215)	0.048	0.167 (0.050–0.285)	0.006
Log U-ET-1 (ng/g creatinine)	0.143 (−0.038 to 0.324)	0.121	−0.028 (−0.258 to 0.203)	0.812	0.099 (−0.154 to 0.353)	0.435

The values presented are adjusted linear regression coefficients (β) and 95 % confidence intervals (CI), estimated by univariate linear regression models with U-ET-1 (logarithm base 10) or with U-TGF-β1 (logarithm base 10) as the dependent variables. The U-ET-1 model was adjusted for 24-h SBP, HOMA-IR, eGFR, aldosterone, U-albumin, U-AGT, log U-TGF-β1, as well as for sex and age (months). The U-TGF-β1 model was adjusted for 24-h SBP, uric acid, eGFR, P-AGT, U-albumin, U-AGT, log U-ET-1, as well as for sex and age (months). The models are presented separately by BMI z-score classes because the effect of U-AGT on each of the urinary cytokines was found to be significant in obese children but not in normal weight children (*p* for interaction=0.047 and 0.049 for urinary ET-1 and TGF-β1, respectively)

SBP, systolic blood pressure; eGFR, estimated glomerular filtration rate

in the progression of kidney disease, promoting fibrosis by stimulation of TGF-β1 [34], but the specific interactions of aldosterone and ET-1 have been less well explored. In animal models, aldosterone has been observed to increase intrarenal ET-1 expression [35] and induce ET-1 release from endothelial cells, contributing to aldosterone-mediated endothelial dysfunction [39]. We cannot rule out the existence of any number of confounding variables not considered in our analysis which could explain our findings, which are at variance with the previously described associations between ET-1 and aldosterone.

In a genetic study of patients with obesity-related glomerulopathy, Wu et al. [38] found that several genes encoding inflammatory cytokines, namely those for TGF-β1, leptin receptors and insulin resistance, were upregulated in the glomeruli, suggesting that IR may directly contribute to the renal injury observed in obesity. Our findings of an association between U-ET-1 and HOMA-IR levels, both in normal weight and obese children, are in line with the results reported by these authors. IR is believed to represent an important pathway for the development of lesions in obesity-related kidney injury [40]. We also found that both U-ET-1 and TGF-β1 levels were positively associated with SBP in fully adjusted models. Although ET-1 is known to be involved in the regulation of vascular tone, being a potent vasoconstrictor peptide released by the endothelium [41], the association of U-ET-1 with BP levels is controversial. Glowinska et al. found higher levels of plasma ET-1 in both obese and hypertensive children, with an association between SBP and ET-1 levels in the latter group [37]. However, other studies evaluating U-ET-1 or U-TGF-β1 failed to find an association between ET-1 levels and BP [33]. Since ET-1 also exerts complex effects on renal water and sodium excretion, depending upon its action on ET_A (antinatriuretic) or ET_B (natriuretic) receptors [42, 43], and there appears to be a gender difference in the renal expression of these receptors [42], the proportion of girls and boys included in the different studies conducted to date is likely to have affected the results.

We acknowledge a number of important limitations to our study, the most important of which is likely our sole reliance on U-AGT levels as a marker of intrarenal RAAS and on the levels of P-AGT and serum aldosterone as components of the systemic RAAS. It would be important to integrate our findings with other RAAS measurements, as well as with measurements of other cytokines already recognized as having a role in obesity-related kidney injury. The determination of fibrogenic cytokine levels in random urine samples may also be a limitation of our study, since the representativeness of the excretion of these markers requires 24-h urine analysis. In our study, 24-h urine samples were collected and stored at −80 °C immediately after delivery at the study site, but the levels of fibrogenic cytokines were not detectable in the pilot samples analyzed. For this reason measurements in random urine samples were performed instead. Despite these limitations, our study is unique and presents innovative results directed toward trying to elucidate some of the intricate mechanisms of obesity-triggered kidney impairment. In addition to revealing an association between urinary fibrogenic cytokines and U-AGT, thanks to the state-of-the-art cardiovascular evaluation of our sample, which included ABPM, we were able to show that high BP levels and IR might also be associated with the expression of fibrogenic cytokines in the kidney.

The association between urinary cytokines and AGT is particularly relevant, attesting to the existence of an early interplay between the RAAS and the process of tissue remodeling which occurs in the kidneys of obese subjects, even before puberty. The possibility of following our sample in future reevaluations of our birth cohort will surely help us to better understand the mechanisms disclosed in this study and to track the evolution of kidney impairment in the setting of obesity throughout their life span.

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Compliance with ethical standards The ObiKid study was approved by the Ethics Committee of Centro Hospitalar São João, E.P.E. and Faculty of Medicine of the University of Porto and complies with the Helsinki Declaration and the current national legislation. Written informed consent from parents (or their legal substitute) and verbal assent from children was obtained, concerning information and biological sample gathering.

Conflicts of interest None of the authors have any financial or nonfinancial competing interests concerning the present study.

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