

Urinary NGAL is a Potential Biomarker for Early Renal Injury in Insulin Resistant Obese Non-Diabetic Children

Running Head: Urinary NGAL in Insulin Resistant Obese Children

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What is already known on this topic?

Neutrophil gelatinase-associated lipocalin (NGAL) is one of the new biomarkers in detecting acute renal injury. NGAL was defined as an early renal injury biomarker in type 2 DM too. Microalbuminuria has significant limitations in determining disease progression. Therefore, identification and validation of new biomarkers for early diagnosis of kidney injury may help to predict nephropathy and progression.

What this study adds?

This study reflects that obese children with normoalbuminuric insulin resistance without diabetes have higher urinary NGAL levels than those without IR and are at risk for early renal damage. Type 2 diabetes has an insidious clinical course. NGAL may be a marker of early renal damage in obese IR children before type 2 diabetes develops.

Abstract

Objective: Neutrophil gelatinase-associated lipocalin (NGAL) is one of the new biomarkers in detecting acute renal injury. There are studies showing the relationship between NGAL and renal injury in obese children. This study aimed to investigate whether the urinary levels of NGAL, kidney injury molecule-1 (KIM-1), and serum cystatin-C are increased in insulin resistance (IR) patients before the development of diabetes.

Methods: Our cross-sectional, case-control study included non-diabetic obese children and adolescent patients with IR and a non-diabetic obese control group with no IR, who applied to the Pediatric Endocrinology outpatient clinic of Manisa Celal Bayar University between 2017-2018. Those with diabetes mellitus, known renal disease were excluded. NGAL and creatinine levels were evaluated in the morning spot urine from all participants. Serum renal functions were evaluated.

Results: Thirty-six control and 63 IR patients were included in the study (68 girls, 29 boys). The mean age of all patients was 13.12 ± 2.64 years and no statistically significant difference was found between the two groups in terms of age and gender. Spot urinary NGAL values in the IR group were higher [median 26.35 ng/mL (range, 7.01-108.7)] than the control group [median 19.5 ng/mL (range, 3.45-88.14)], statistically significant higher ($p = 0.018$). NGAL/creatinine ratio was also significantly higher in the IR group compared to the control group ($p = 0.018$).

Conclusions: Obese pediatric patients with IR were shown to have renal injury. Urinary NGAL examination may show early renal injury before development of diabetes.

Keywords: NGAL, renal injury, child, KIM-1, insulin resistance.

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Introduction

The impact of insulin resistance (IR) and obesity on chronic kidney disease (CKD) have been demonstrated [1-4]. Obesity is an important driver of microvascular dysfunction (MVD) [5]. The relationship between hyperglycemia and MVD is bidirectional and constitutes a vicious cycle. Experimental data supported that hyperglycemia causes microvascular disease [6]. MVD contributes to IR and the onset of type 2 diabetes mellitus (T2DM) [5] with a higher prevalence of comorbidities in youth [5-7]. Also, the reduction of hyperglycemia is associated with a reduction of onset and progression of nephropathy. MVD precedes nephropathy [8-10].

The diagnostic standard noninvasive test currently used in clinical practice to predict the onset and monitor the progression of diabetic nephropathy is microalbuminuria measurement. However, this is a sign of early glomerular damage rather than a marker for susceptibility to it. Microalbuminuria has significant limitations in determining disease progression because of the observation that some type 1 diabetes mellitus (T1DM) patients revert to normoalbuminuria without treatment [11]. Also, studies assert that tubulointerstitial injury may precede the appearance of glomerulopathy in diabetic nephropathy [12-13]. Therefore, identification and validation of new biomarkers for early diagnosis of kidney injury may help to predict nephropathy and progression [14]. Tubular injury biomarkers such as urinary NGAL, urinary KIM-1, serum cystatin C, urinary IgG, transferrin were investigated in pediatric and adult patients with T2DM [14-8]. NGAL was defined as an early

renal injury biomarker in type 2 DM [19]. The question of our study is whether there is early renal damage before the development of diabetes in obese children with IR showed by this biomarkers.

Methods

Participants:

This single-center, cross-sectional, case-control study included children aged between 7-18 years, who applied Manisa Celal Bayar University Hospital, Pediatric Endocrinology and Pediatric outpatient clinic with the complaint of obesity, between June 1, 2017, and May 31, 2018. We divided the patients into two groups; IR group and control group.

Children with type 1 diabetes or obesity with a syndrome (Prader-Willi syndrome, Laurence Moon Biedl syndrome, etc.) or endocrinologic or metabolic pathologies, dietary supplementation were excluded. Children with infection, kidney or other systemic diseases were also excluded from the study. None of the children were using antihypertensive and antilipidemic drugs.

Procedures

1-Clinical and laboratory evaluation

All obese/overweight patients underwent a thorough physical examination and routine laboratory evaluation, including obesity screening tests (TSH, fT4, fasting glucose, fasting insulin, HOMA-IR), urinalysis, and urinary culture. The assessments were all performed by a single, specially trained clinical research staff. Demographic information, urinary tract abnormalities, urinary tract infections were questioned.

The children and their families were informed about the study and written informed consent was obtained. The local ethics committee (Manisa Celal Bayar University/2015-20478486-217) approved the study in accordance with the Declaration of Helsinki.

2-Classification

We calculated the BMI as weight (kg) divided by square of height (m²). BMI-SDS and BMI percentiles were calculated using age and gender-specific norms published by Neyzi 2006 [20]. Obesity was defined as BMI \geq 95th percentile, and overweight was defined as BMI \geq 85th for age and sex [21].

Insulin resistance was evaluated according to the homeostasis model assessment insulin resistance (HOMA-IR) index which was calculated using the following formula: [Fasting insulin (mU/mL) x Fasting glucose (mg/dL) / 405] [22]. Cut-off values for different stages were prepubertal > 2.5, pubertal > 4 [23].

Prediabetes was defined according to hemoglobin A1c 5,7-6,4% or fasting plasma glucose levels 100-126 mg/dL and/or two-hour plasma glucose levels 140-199 mg/dL according to oral glucose tolerance test (OGTT) [24].

Testing for diabetes was done by measuring hemoglobin A1c > 6,5 % with a random glucose level > 200 mg/dL or fasting plasma glucose > 126 mg/dL or by performing an OGTT, post OGTT 2-hour plasma glucose level > 200 mg/dL [24]. Patients with diabetes were excluded.

Blood pressure was taken with the appropriate cuff, systolic blood pressure (SBP), and diastolic blood pressure (DBP) were measured twice, after a ten-minute rest, using the right arm and a calibrated sphygmomanometer and the mean of these two BP values were calculated. Hypertension was defined as a value above the 95th percentile for age and height, according to the National Health and Nutrition Examination Survey [25].

An ambulatory blood pressure monitoring (ABPM) device was applied on the same day. ABPM protocol was performed by a single investigator (Ç.Ö). A validated recorder (Contec ABPM50, Germany), was programmed to measure BP at 20-min intervals from 8 AM to 11 PM and at 30-min intervals from 11 PM to 8 AM. The most appropriate original standard cuff was selected depending upon the individual's non-dominant arm. The participants were instructed to follow their usual daily activities, to avoid strenuous exercise and shower, to remain still with the forearm extended during measurements, to note the time when they went to bed and arose, and to detach the device 24 h later. Measurements with systolic BP < 240 and > 70 mm Hg, diastolic BP < 140 and > 40 mm Hg, and diastolic BP < systolic BP were accepted as valid [26]. ABPM data with < 30 valid daytime BP measurements and/or < 12 nighttime measurements were not included [27].

Microalbumin levels were measured in a 24-hour urine sample of all participants. Microalbuminuria was defined as a urinary albumin 30–300 mg/24 hours. Microalbuminuric patients were examined for three times. If one result is negative we classified it as normoalbuminuria [28].

3- Laboratory measurements:

All blood samples were collected in the morning after at least 8 hours fast (at least 8 hours) for measurements of complete blood count and biochemical parameters, including obesity screening tests (fasting glucose, fasting insulin, HOMA-IR, TSH, fT4, total cholesterol (TC), low-density lipoprotein cholesterol (LDL-C), high-density lipoprotein cholesterol (HDL-C), triglyceride), urea, creatinine (Cr), cystatin C. Estimated glomerular filtration rate (eGFR) was calculated using the Schwartz formula [29].

The morning spot urine samples (at least 10 mL) were collected from all patients and centrifuged (3000 rpm for five minutes), and the supernatants were frozen at -80°C until analysis. Urinary NGAL (u-NGAL), urinary KIM-1 were measured (ELISA, Human NGAL Platinum ELISA Kit (eBioscience™, Vienna, Austria)). Urine samples were diluted according to the kit package insert. Urinary NGAL levels were obtained by multiplying the results with 1000 dilution factor. The kit sensitivity is 6.5 pg/mL. The average intra-assay precision CV value of the kit was calculated as 4.0%. KIM-1 levels in human urine samples were analyzed by Human KIM-1 ELISA Test Kit (BioAssay Works, Ijamsville, USA) with the Enzyme-Linked Immunoassay (ELISA) method. Urine samples were diluted according to the kit package insert. The obtained results were multiplied by six dilution factor, and urinary KIM-1 levels were obtained.

Statistical analysis

The sample size was calculated using the power analysis method; an effect size of 0.70 and a power of 0.80 (alpha 0.05) required a sample of 63/36 people for a case/control study.

Comparison of study variables was first made between patients with IR and control group. Between-group comparison for categorical variables was performed by using the χ^2 test, or Fisher's exact tests. All data were tested for normality using the Kolmogorov-Smirnov test or Shapiro-Wilk. Mann-Whitney U test was used for comparison of not normally distributed

continuous and nonparametric variables, T-test was used for normally distributed variables. Univariate correlation analysis was performed between IR participants and healthy controls using Spearman test. Statistical analyses were performed using Statistical Package for Social Sciences 15.0 (SPSS 15.0, Chicago, IL, USA) program. P values <0.05 were considered statistically significant.

Results

In this study, 99 obese (n=95) and overweight (n=4) children/adolescents were evaluated. The median age of the participants was 13 years (range 7-18) and 68 (68.7%) were female. Our first group consisted of 63 participants with IR (male/female: 17/46; age 13,9 years (range 7-18)). The second group consisted of 36 obese controls without IR (male/female: 14/22; age 12,1 years (8-17)). No statistically significant difference was found between two groups in terms of age, gender and BMI (Table 1). The number of prediabetics was higher in IR group than control group (20 vs. 3) (p = 0.008). Insulin resistance group and control group were similar according to hypertension (17 vs. 9; p=0,908). Comparison of ABPM data among the two groups resulted in similar hypertension rates and nighttime BP standard deviation score (SDS) values. None of the hypertensives were found to have a secondary cause. The percentages of systolic and diastolic dipping and the rate of non-dippers were similar between the two groups. None of the participants had leukocytosis, neutrophilia, thrombocytosis, elevated creatinine or abnormal GFR levels (according to Schwartz) or hypothyroidism. Remaining laboratory analyses are shown in Table 1.

Microalbuminuria median levels were similar in both groups IR (p=0,252). Although urinary KIM-1, KIM-1/cre, serum cystatin-C levels were similar between the groups, median urinary NGAL levels [median 26,35 ng/mL (range, 7,01-108,7) vs. 19,5 ng/mL (range, 3,45-88,14), p=0,018] and NGAL/cre levels [median 0,27 (range, 0,05-1,58) vs. 0,16 (range, 0,01-1,5), p=0,018] were higher in IR group compared with control group (Table 1).

Discussion

In daily practice, microalbuminuria is used for renal injury in diabetes. We investigated whether the prediction of renal injury is possible in the IR stage with biomarkers such as serum cystatin C, urinary KIM-1, and urinary NGAL before diabetes developed. This study showed that NGAL examination may be used as an early biomarker for renal injury in obese IR patients in the absence of diabetes and microalbuminuria. However, there was no significant difference in cystatin and urinary KIM-1 values.

The increasing worldwide rates of obesity in children and adolescents is strongly associated with IR, which in turn is related to some problems, including T2DM [30]. Patients with youth-onset T2DM are at considerable risk for diabetic nephropathy and eventually, renal failure in young adulthood due to microvascular complications [31-3]. Because of the insidious onset of T2DM, the real duration of the disease is often not known; therefore, there is a lower correlation of albumin excretion rate with disease duration than in T1DM [30]. A considerable number of patients with microalbuminuria present at T2DM diagnosis. Screening for microalbuminuria should begin at the time of diagnosis and continue annually [30]. Compared with T1DM, in young-onset T2DM microalbuminuria was observed more frequent with earlier and rapid progression to diabetic nephropathy due to IR [7, 32, 34-7]. Our findings support this hypothesis. IR may be the starting point of diabetic nephropathy. This study has shown the tubular kidney damage in obese children with IR. Based on this findings, patients may need to be screened with tubular biomarkers at the IR stage before T2DM develops. Experimental studies have shown that reduced insulin sensitivity and hyperinsulinemia are important factors leading to renal injury. It has been expected that insulin influences renal function primarily at the tubular level, as specific binding of insulin is greatest in thick ascending limb and distal convoluted tubules [38]. There were also studies supporting that insulin acts in the proximal tubules [39]. The experimental number of studies have shown that hyperinsulinemia led to decreased nitric oxide levels, increased transforming growth factor- β 1 (TGF- β 1), insulin-like growth factor-1 (IGF-1), endothelin-1 production and increased oxidative stress [40-4]. These studies could be an explanation for our results.

Neutrophil gelatinase-associated lipocalin is a member of the lipocalin family. Several studies suggest that NGAL might be a marker for a variety of diseases associated with lipid metabolism such as obese-inflammation-induced metabolic syndrome, IR, glucose and lipid metabolism or endothelial dysfunction [45]. NGAL is known to be released from injured renal tubular cells in acute kidney injury before a decrease in the GFR can be detected [46]. Furthermore, urinary NGAL can be used as an early biomarker of diabetic nephropathy [47]. The results of a meta-analysis, which also included pediatric studies, suggest that NGAL in urine can be considered a valuable biomarker for early detection of diabetic nephropathy in the normoalbuminuric stage. It is well known that the pathophysiology and progression of diabetic nephropathy were associated with both glomerular and tubular interstitial damage, and it has been shown that in the absence of glomerular proteinuria, tubular dysfunction can even precede glomerular injury and, thus, microalbuminuria. In recent studies, NGAL concentrations were found to be increased in patients with type 2 diabetes mellitus with or without albuminuria in subclinical tubular damage [14, 19, 48-9]. However, most childhood studies emphasize the relationship between NGAL and tubular damage in the T1DM population. [50-4]. Also, it was shown that normal range albuminuria does not exclude nephropathy in type 1 diabetic children [55]. This study is the first publication showing the relationship between renal tubular damage and NGAL in childhood with IR before T2DM development.

In adult T2DM patients, urinary KIM-1 levels were found to be significant in showing renal impairment [56-8]. In a study of obese children, urinary KIM-1 was shown to be not significant for determining renal injury [59]. Similarly to KIM-1, studies have demonstrated that cystatin C was a useful marker of early renal impairment in T2DM adults. In a recent pediatric study, Cystatin C levels were shown to be higher in obese with metabolic syndrome (MetS) compared to those without MetS. In our study, obese children with IR and without IR had similar urinary KIM-1 and serum cystatin C levels [60-1]. These findings suggest that NGAL shows renal effects earlier than KIM-1 and cystatin C.

Limitations

There are some limitations to our study. The number of our control group was low. We could not see if the renal effects of children with IR were reversible by treatment. Due to the insidious nature of the IR, we were unable to determine the IR

exposure times of children and their contribution to renal exposure. The gold standard is a biopsy in terms of renal affection, but it is not possible to do it due to the ethical aspect.

Conclusions

In obese children with normoalbuminuric IR without T2DM, urinary NGAL levels are higher than those without IR and are at risk for early renal damage. Due to the insidious course of T2DM, attention should be paid to renal damage from the IR stage. To the best of our knowledge, this is the first pediatric study showing the tubular damage with NGAL in IR.

Declaration of competing interest

No author reported a conflict of interest.

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Table 1: Sociodemographic characteristics, laboratory findings, urinary NGAL, urinary KIM-1 and serum cystatin-C levels of obese insulin resistance group and control group

	Insulin Resistance group (n=63)	Control group (n=36)	<i>p</i>
Age median (range) (years)	13,9 (7-18)	12,1 (8-17)	0,061
Gender (M/F)	17/46	14/22	0,263
BMI (kg/m ²)	29,8 (17-42)	28,1 (20-39)	0,063
SBP (mmHg) median (range)	115 (80-150)	115 (90-160)	0,923
DBP (mmHg) median (range)	70 (50-100)	70 (50-100)	0,587
Prediabetes (n)	20	3	0,008
Fasting glucose median (range) (mg/dL)	86 (67-105)	84 (70-100)	0,486
Fasting insulin median (range) (mIU/L)	30,30 (17-60)	16,05 (8-21)	0,000
HOMA-IR	6,06 (4-14)	3,3 (2-4)	0,000
Blood Urea median (range) (mg/dL)	21,7 (13-36)	24(15-34)	0,402
Blood creatinine (mg/dL) median (range)	0,56 (0,1-1)	0,5(0,1-1)	0,717
Uric acid (mg/dL)	5 (1,60-9,60)	4,85(3,20-7,10)	0,155
eGFR median (range) (Schwartz)	119,5 (83,50-231)	120,20(70,7-178,10)	0,608

(mL/min/1.73m ²)			
Triglycerides(mg/dL)	114 (44-389)	96 (42-257)	0,167
Total cholesterol (mg/dL)	165 (103-254)	149,5 (91-219)	0,062
HDL-c (mg/dL)	45,1 (32-79)	47,05 (26-68)	0,417
LDL-c (mg/dL)	92 (19-187)	78,5 (34-131)	0,077
TSH (mIU / L)	2,5 (1,05-5,8)	2,55 (0,95-5,6)	0,954
Urinary NGAL median (range) (pg/mL)	26,35 (7,01-108,7)	19,5 (3,45-88,14)	0,018
NGAL/cre median (range) pg/mg	0,27 (0,05-1,58)	0,16 (0,01-1,5)	0,018
KIM-1 median (range) (pg/mL)	0,84 (0-2,09)	0,85 (0-6,18)	0,789
KIM-1/cre median (range)	0,01 (0,00-0,03)	0,008(0,00-0,06)	0,570
Cystatin C (mg/L) median (range)	0,82 (0,28-1)	0,84 (0,7-1)	0,154
Cystatin C eGFR median (range) <i>mL/dk/1.73 m²</i>	93,7(72,20-170,4)	90,75(71,5-110,5)	0,138
eGFR median (range)	151,05(80,3-361,1)	180,8(85,7-417,02)	0,141
Urinary protein/creatinine Median mg/gr (range)	0,04 (0,02-0,16)	0,04 (0,02-0,61)	0,994
Microalbuminuria mg/24 h median (range)	6 (0-29)	6,8 (0-29,9)	0,252

BMI, body mass index; DBP, diastolic blood pressure; GlcT0¹, fasting glucose; HDL-c, high density lipoprotein cholesterol; HOMA-IR, homeostatic model assessment of insulin resistance; InsT0¹, fasting insulin; KIM-1: kidney injury molecule-1; LDL-c, low density lipoprotein cholesterol; NGAL: Neutrophil gelatinase-associated lipocalin; SBP, systolic blood pressure; SDS, standard deviation score; TG, triglycerides.