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

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What is already known on this topic?

Neutrophil gelatinase-associated lipocalin (NGAL) is a newly described biomarker used for detecting acute renal injury. NGAL was also defined as an early renal injury biomarker in type 2 diabetes mellitus. Type 2 diabetes has an insidious clinical course. 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?

Obese children with normoalbuminuric insulin resistance (IR) without diabetes have higher urinary NGAL levels than those with no evidence of IR and are at risk for early renal damage. 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 for detecting acute renal injury. There are studies showing the relationship between NGAL and renal injury in obese children. The aim of this study was to investigate whether urinary levels of NGAL, kidney injury molecule-1, and serum cystatin C are increased in insulin resistance (IR) patients before the development of diabetes.

Methods: Cross-sectional, case-controlled study that included non-diabetic obese children and adolescent patients with IR and a non-diabetic obese control group with no IR, who attended a tertiary center pediatric endocrinology outpatient clinic between 2016-2018. Those with diabetes mellitus and/or known renal disease were excluded. NGAL and creatinine (Cr) levels were evaluated in the morning spot urine from all participants. Serum renal function was evaluated.

Results: Thirty-six control and 63 IR patients were included in the study, of whom 68 (68.7%) were girls. The mean age of all participants was 13.12 ± 2.64 years and no statistically significant difference was found between the two groups in terms of age or gender distribution. Median (range) spot urinary NGAL (u-NGAL) values in the IR group were significantly higher at 26.35 (7.01-108.7) ng/mL than in the control group at 19.5 (3.45-88.14) ng/mL (p = 0.018). NGAL/Cr ratio was also significantly higher in the IR group compared to the control group (p = 0.018).

Conclusion: Obese pediatric patients with IR were shown to have elevated levels of u-NGAL, a marker of renal injury. u-NGAL examination may show early renal injury before development of diabetes.

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

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Şen S et al.
Urinary Neutrophil Gelatinase-associated Lipocalin in Insulin Resistant Obese Children

Introduction

The impact of insulin resistance (IR) and obesity on chronic kidney disease has been reported (1,2,3,4). Obesity is an important driver of microvascular dysfunction (MVD) (5). The relationship between hyperglycemia and MVD is bidirectional and can be considered a vicious circle. Experimental data suggest that hyperglycemia may cause 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 (5,6,7). In addition, a reduction in hyperglycemia is associated with delay of onset and reduced progression of nephropathy. MVD precedes nephropathy (8,9,10).

The standard noninvasive diagnostic 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 suggest 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). Biomarkers of tubular injury, such as urinary neutrophil gelatinase-associated lipocalin (NGAL), urinary kidney injury molecule 1 (KIM-1), serum cystatin C, urinary IgG, and transferrin have been investigated in pediatric and adult patients with T2DM (14,15,16,17,18). NGAL has been shown to be an early renal injury biomarker in type 2 DM (19). The aim of the present study was to investigate whether levels of these biomarkers, suggesting early renal damage, are elevated in obese children with IR before the development of diabetes.

Methods

Participants

This single-center, cross-sectional, case-control study included children aged between 7-18 years, who attended Manisa Celal Bayar University Hospital, Pediatric Endocrinology and Pediatric Outpatient Clinics with the complaint of obesity, between January 1, 2016, and May 31, 2018. Patients were divided into two groups: those with IR (IR group) and those without IR (control group).

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

Clinical and Laboratory Evaluation

All obese/overweight patients underwent a thorough physical examination and routine laboratory evaluation, including obesity screening tests, urinalysis, and urinary culture. Obesity screening tests included measurement of blood thyroid stimulating hormone (TSH), free thyroxine (fT4), fasting glucose, fasting insulin, lipid profile and estimation of homeostasis model assessment-IR (HOMA-IR). These assessments were all performed by a single, specially-trained clinical researcher. Demographic information was collected and urinary tract abnormalities and urinary tract infections were investigated.

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

Classification of Patients

Body mass index (BMI) was calculated using the standard formula; weight (in kg) divided by square of height in meters (m^2). BMI standard deviation score (SDS) and BMI percentiles were calculated using age and gender-specific norms published by Neyzi et al 2006 (20). Obesity was defined as BMI ≥95th percentile, and overweight was defined as BMI ≥85th for age and sex (21).

IR was evaluated according to the 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 and pubertal >4 (23).

Prediabetes was defined according to hemoglobin A1c (HbA1c) in the range 5.7-6.4% or fasting plasma glucose levels 100-126 mg/dL and/or two-hour plasma glucose levels 140-199 mg/dL following an oral glucose tolerance test (OGTT) (24).

Testing for diabetes was done by measuring HbA1c, with an HbA1c >6.5% concurrent with a random glucose level >200 mg/dL or fasting plasma glucose >126 mg/dL indicating diabetes. Alternatively, by performing an OGTT, with a post-OGTT 2-hour plasma glucose level >200 mg/dL also indicating diabetes (24). Patients with diabetes were excluded.

Şen S et al.
Urinary Neutrophil Gelatinase-associated Lipocalin in Insulin Resistant Obese Children

J Clin Res Pediatr Endocrinol
2021;13(4):400-407

Blood pressure was taken with the appropriate cuff, systolic blood pressure, and diastolic blood pressure 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 used 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 hours 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 obtained from all participants. Microalbuminuria was defined as a urinary albumin 30-300 mg/24 hours. Microalbuminuric patients were examined three times. If one result was negative, it was subsequently classified as normoalbuminuria (28).

Laboratory Measurements

All blood samples were collected in the morning after at least 8 hours of fasting for measurements of complete blood count and biochemical parameters, including obesity screening tests, urea, creatinine (Cr), and cystatin C. Obesity screening tests included fasting glucose, fasting insulin, HOMA-IR, TSH, fT4, total cholesterol, low-density lipoprotein cholesterol, high-density lipoprotein cholesterol, and triglyceride measurements. 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) was measured by enzyme-linked immunosorbent assay (ELISA) with a commercially available kit (Human NGAL Platinum ELISA Kit, eBioscience,

Vienna, Austria). Urine samples were diluted according to the kit package insert. u-NGAL levels were obtained by multiplying the results by 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 using the Human KIM-1 ELISA Test Kit (BioAssay Works, Ijamsville, USA). Urine samples were diluted according to the kit package insert. The obtained results were multiplied by the dilution factor (a six-fold multiplication).

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-Wilks test. Mann-Whitney U test was used for comparison of non-normally distributed continuous and nonparametric variables, while the t-test was used for normally distributed variables. Univariate correlation analysis was performed between IR participants and healthy controls using the Spearman test. Statistical analyses were performed using Statistical Package for Social Sciences, version 15.0 (IBM Inc., Chicago, IL, USA). A $p < 0.05$ was considered statistically significant.

Results

In this study, 99 obese and overweight children/adolescents were evaluated, of whom 95 (96%) were classified as obese and the remaining four were overweight. The median (range) age of the participants was 13 (7-18) years and 68 (68.7%) were female. Patients were divided into two groups based on the presence of IR or not. The group with IR consisted of 63 participants (male/female: 17/46) with a median (range) age of 13.9 (7-18) years. The group without IR consisted of 36 obese controls (male/female: 14/22) with a median (range) age of 12.1 (8-17) years. These two groups were similar in terms of age, gender and BMI (Table 1). Prediabetes was significantly more common in the IR group than the control group (20 vs. 3; $p = 0.008$). There was no difference between the IR group and the control group in terms of prevalence of hypertension (17 vs. 9, respectively; $p = 0.908$). Comparison of ABPM data between the two

J Clin Res Pediatr Endocrinol
2021;13(4):400-407

Şen S et al.
Urinary Neutrophil Gelatinase-associated Lipocalin in Insulin Resistant Obese Children

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

	Insulin resistance group (n = 63)	Control group (n = 36)	p
Age (years)	13.9 (7-18)	12.1 (8-17)	0.061
Gender (M/F) n (%)	17/46 (26.9/73.1)	14/22 (38.8/61.2)	0.263
BMI (kg/m^2)	29.8 (17-42)	28.1 (20-39)	0.063
SBP (mmHg)	115 (80-150)	115 (90-160)	0.923
DBP (mmHg)	70 (50-100)	70 (50-100)	0.587
Prediabetes n (%)	20	3	0.008
Fasting glucose (mg/dL)	86 (67-105)	84 (70-100)	0.486
Fasting insulin (mUI/L)	30.30 (17-60)	16.05 (8-21)	< 0.001
HOMA-IR	6.06 (4-14)	3.3 (2-4)	< 0.001
Blood urea (mg/dL)	21.7 (13-36)	24.0 (15-34)	0.402
Blood creatinine (mg/dL)	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 (Schwartz) (mL/min/1.73 m^2)	119.5 (83.50-231)	120.2 (70.7-178.1)	0.608
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 (pg/mL)	26.35 (7.01-108.7)	19.5 (3.45-88.14)	0.018
NGAL/creatinine ratio (pg/mg)	0.27 (0.05-1.58)	0.16 (0.01-1.5)	0.018
KIM-1 (pg/mL)	0.84 (0-2.09)	0.85 (0-6.18)	0.789
KIM-1/creatinine ratio	0.01 (0-0.03)	0.008 (0-0.06)	0.570
Serum cystatin C (mg/L)	0.82 (0.28-1.0)	0.84 (0.7-1.0)	0.154
Cystatin-C eGFR median	93.7 (72.2-170.4)	90.75 (71.5-110.5)	0.138
Urinary protein/creatinine (mg/g)	0.04 (0.02-0.16)	0.04 (0.02-0.61)	0.994
Microalbuminuria (mg/24 hours)	6 (0-29)	6.8 (0-29.9)	0.252

BMI: body mass index, DBP: diastolic blood pressure, 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, M/F: male/female, eGFR: estimated glomerular filtration rate, TSH: thyroid stimulating hormone

groups resulted in similar hypertension rates and nighttime BP SDS values. None of the subjects with hypertension 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 Cr or abnormal eGFR levels or hypothyroidism. The remaining laboratory analyses are shown in Table 1.

Median urinary microalbumin levels were similar in both groups (p = 0.252). Urinary KIM-1, KIM-1/Cr ratio, and serum cystatin C levels were similar between the IR and control groups. However median (range) u-NGAL levels and NGAL/Cr ratios were significantly greater in the IR group compared to the control group at 26.35 (7.01-108.7) vs. 19.5 (3.45-88.14) ng/mL (p = 0.018) and 0.27 (0.05-1.58) vs. 0.16 (0.01-1.5) (p = 0.018), respectively (see Table 1).

Discussion

In daily practice, measurement of microalbuminuria is used to screen and monitor renal injury in diabetes. This study investigated whether the prediction of renal injury is possible at the IR stage using biomarkers, such as serum cystatin C, urinary KIM-1, and u-NGAL, before diabetes has developed. The results suggest that NGAL measurements may be used as an early biomarker for renal injury in obese patients with IR in the absence of diabetes and microalbuminuria. However, the other biomarkers examined, serum cystatin C and urinary KIM-1, did not differ between the IR group and the non-IR controls in this study.

The increasing global rates of obesity in children and adolescents is strongly associated with IR, which in turn is associated with some conditions, including T2DM (30). Patients with youth-onset T2DM are at considerable risk for diabetic nephropathy and. eventually, renal failure in young

Şen S et al.
Urinary Neutrophil Gelatinase-associated Lipocalin in Insulin Resistant Obese Children

J Clin Res Pediatr Endocrinol
2021;13(4):400-407

adulthood due to microvascular complications (31,32,33). As the onset of T2DM may be insidious in many cases, the real duration of the disease is often not known. This may be one of the reasons why there is a poorer correlation between albumin excretion rate and disease duration in T2DM compared to T1DM (30). At T2DM diagnosis, many patients already have microalbuminuria. Screening and monitoring of microalbuminuria should begin at the time of diagnosis and continue annually (30). Compared to T1DM, in young-onset T2DM microalbuminuria was observed more frequently, with earlier and rapid progression to diabetic nephropathy, due to IR (7,32,34,35,36,37). Our findings support this study results. IR may be the starting point of diabetic nephropathy. Our results suggest the presence of tubular kidney damage, as evidenced by elevated NGAL levels, in obese children with IR. Based on this finding, patients may need to be screened for biomarkers of tubulopathy at the IR stage, before T2DM develops. Experimental studies have shown that reduced insulin sensitivity and hyperinsulinemia are important factors leading to renal injury (38). It is accepted that insulin mediates renal function, primarily at the tubular level, as specific binding of insulin is greatest in the thick ascending limb and distal convoluted tubules (39). There is also evidence that insulin acts in the proximal tubules (40). A number of experimental studies have shown that hyperinsulinemia led to decreased nitric oxide levels, increased transforming growth factor-β1 and insulin-like growth factor-1 levels, and endothelin-1 production, resulting in increased oxidative stress (38,41,42,43,44). These mechanisms may explain the increased NGAL found in our IR group, which also had significantly elevated levels of fasting insulin compared to the control group.

NGAL is a member of the lipocalin family. Several studies suggest that NGAL might be a marker for a variety of conditions associated with lipid metabolism, such as obese-inflammation-induced metabolic syndrome (MetS), IR, disrupted glucose and lipid metabolism or endothelial dysfunction (45). It has been shown that NGAL is released from injured renal tubular cells in acute kidney injury before a decrease in the GFR can be detected (46). Furthermore, u-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 accepted that the pathophysiology and progression of diabetic nephropathy are 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 concentration was found to be increased in patients with T2DM, with or without albuminuria in subclinical tubular damage (14,19,48,49). However, most childhood studies have investigated the relationship between NGAL and tubular damage in T1DM (50,51,52,53,54). Also, it was shown that normal range albuminuria does not exclude nephropathy in children with T1DM (55). The present study is the first to demonstrate a relationship between renal tubular damage and NGAL in children with IR before T2DM development.

In adult T2DM patients, urinary KIM-1 levels were found to be a useful biomarker of renal impairment (56,57,58). However, in a study of obese children, urinary KIM-1 was shown to not be associated with renal injury (59). Similarly, studies in T2DM adults have demonstrated the clinical utility of measuring cystatin C as a useful marker of early renal impairment. In children cystatin C levels were shown to be elevated in obese subjects with MetS compared to those without MetS (60,61). The similarity in median urinary KIM-1 and serum cystatin C levels in the IR and control groups, while finding significant differences in median u-NGAL concentrations, suggest that NGAL is an earlier marker of renal effects in IR associated with pediatric obesity.

Study Limitations

There are some limitations to our study. The number of participants in the control group was low. The reversibility of the observed renal effects by treatment was not investigated. It was also not possible to estimate the duration of IR, and therefore its possible relationship to changes in the measured biomarkers in affected subjects. The gold standard for assessment of renal damage is a biopsy, but performance of renal biopsy in this study was not ethically acceptable.

Conclusion

In obese children with normoalbuminuric IR without T2DM, u-NGAL levels were significantly higher than in obese children without IR. This suggests that the former are at risk of early renal damage. To the best of our knowledge, this is the first pediatric study showing evidence of tubular damage, indicated by elevated NGAL in children with IR. We suggest that, because of the insidious onset of T2DM, screening for renal damage should be performed from the IR stage and urinary NGAL may be a useful marker for this. Further studies are needed to investigate the relationship between u-NGAL and definitive measures of renal damage, such as renal biopsy, in children with IR.

J Clin Res Pediatr Endocrinol
2021;13(4):400-407

Şen S et al.
Urinary Neutrophil Gelatinase-associated Lipocalin in Insulin Resistant Obese Children

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Şen S et al.
Urinary Neutrophil Gelatinase-associated Lipocalin in Insulin Resistant Obese Children

J Clin Res Pediatr Endocrinol
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J Clin Res Pediatr Endocrinol
2021;13(4):400-407

Şen S et al.
Urinary Neutrophil Gelatinase-associated Lipocalin in Insulin Resistant Obese Children

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