Neutrophil Gelatinase-Associated Lipocalin and Retinol-Binding Protein-4 as Biomarkers for Diabetic Kidney Disease

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Keywords
Neutrophil gelatinase-associated lipocalin · Retinol-binding protein 4 · Diabetes type 2 · Albuminuria · Chronic kidney disease

Abstract
Aims: This study was designed to evaluate the conflicting association between 2 tubular protein markers including neutrophil gelatinase-associated lipocalin (NGAL) and retinol-binding protein-4 (RBP-4) with albuminuria and glomerular filtration rate (GFR) and calculate the accuracy of the role of NGAL and RBP-4 in diagnosis of diabetic nephropathy (DN) in patients with type2 diabetes. Methods: This is a cross-sectional study that included 133 patients with type 2 diabetes. There were 3 diabetic study groups with normoalbuminuria, moderately increased albuminuria, and severely increased albuminuria, and non-diabetic control group without any renal disease. We analyzed the difference of urinary NGAL (uNGAL) and RBP-4 between nondiabetics and diabetics, as well as within the diabetic group. We also assessed the association between albuminuria and NGAL and RBP-4. Results: The urinary levels of NGAL and RBP-4 were higher in patients with type 2 diabetes compared to nondiabetics as well as in albuminuric diabetics, as well as within the diabetic group. We also assessed the association between albuminuria and NGAL and RBP-4. Conclusion: NGAL and RBP-4 are potential markers of tubular damage that may increase before the onset of glomerular markers such as albuminuria and GFR in patients with type 2 diabetes. Therefore, these markers can be used as complementary measurements to albuminuria and GFR in the earlier diagnosis of DN.
Introduction

Diabetic kidney disease (DKD), which is one of the most common microvascular complications of diabetes, accounts for not only the leading cause of end-stage renal disease but also causes major morbidity and mortality in patients with type 2 diabetes [1–3]. In Iran, the prevalence of nephropathy among the clinically registered adults with diabetes was estimated to be 16.70% in women and 17.60% in men [4]. Recent studies have represented that diabetic nephropathy (DN) can begin even in the early stage of diabetes [5]. Although albuminuria and glomerular filtration rate (GFR) are currently accepted standards for diagnosing the onset or progression of DN, some patients with DKD at early stages may show normal urinary albumin level or normal estimated GFR (eGFR) [6]. These conflicting ideas about a diagnostic marker for early detection of renal pathological changes in diabetes have prompted the consideration of the role of other biomarkers. So that, multiple tubular damage markers currently have been detected that could contribute to the early pathogenesis of DKD [2]. Our study was conducted to evaluate 2 of these biomarkers which are increased in response to tubular injury such as neutrophil gelatinase-associated lipocalin (NGAL) and retinol-binding protein 4 (RBP-4).

NGAL is a 25 kDa protein from the lipocalin family. It is produced by neutrophils and injured nephron epithelial cells. NGAL is specifically released into blood and urine as a reaction to nephron injury. Higher urinary levels of NGAL were reported in patients with diabetes in comparison to healthy nondiabetic individuals [6]. Several studies have suggested that NGAL is a specific marker for acute kidney injury as well as early detection of DN [7–9].

RBP-4 is also a 21 kDa protein belonging to the lipocalin superfamily which is almost completely reabsorbed by proximal tubular cells after glomerular filtration. Therefore, urinary RBP-4 has been identified as a very sensitive biomarker for proximal tubular cells dysfunction. Previous studies have shown not only increasing serum and urinary levels of RBP-4 in patients with diabetes but also a determining relationship between urinary RBP-4 and the progression of chronic kidney disease (CKD) in the severely increased albuminuric patients with diabetes. However, the occurrence of normal and moderately increased albuminuria with RBP-4 levels is yet controversial [1].

Most of previous studies have shown a positive correlation between urinary levels of potential tubular injury markers such as NGAL and RBP-4 and glomerular injury markers such as albuminuria [1, 8, 10–15], but there are conflicting reports about the correlation between NGAL and RBP-4 levels with GFR. On the other hand, some studies have shown a significant negative correlation between NGAL and GFR, while others have revealed an independent relationship between these 2 [16–21]. The controversies among the previous studies urged us to measure the levels of urinary NGAL (uNGAL) and RBP-4 in patients with type 2 diabetes who had normal, moderately increased, or severely increased albuminuria. We aimed to explore the relationship between NGAL and RBP-4 as tubular injury markers with GFR and different stages of diabetic albuminuria as well as evaluating the accuracy of these 2 tubular injury markers in diagnosing CKD in patients with type 2 diabetes.

Material and Methods

Study Population

This cross-sectional study was conducted in the Diabetes Clinic of Vali-Asr Hospital, a teaching hospital affiliated with Tehran University of Medical Sciences (Tehran, Iran) from 2018 to 2019. One hundred and thirty-three patients with diabetes were recruited...
and categorized into 3 groups: 44 patients with normal albumin excretion (albumin/creatinine ratio [ACR] < 30 mg/g creatinine [Cr]), 45 patients with moderately increased albuminuria (ACR 30–300 mg/g Cr), and 44 patients with severely increased albuminuria (ACR > 300 mg/g Cr). Moreover, we recruited 39 healthy participants as the control group. The diagnosis of diabetes was made based on the American Diabetes Association diagnostic criteria (2014). We included patients with type 2 diabetes with no other cause for nephropathy other than diabetes, no inflammatory, rheumatologic or neoplastic illness, no use of glucocorticoids, without symptomatic heart failure, with normal liver function and with Cr > 2. Exclusion criteria were smoking, pregnancy, congestive heart failure, hypertension or use of anti-hypertension medications, alternation in leukocyte count, renal transplant, severe liver dysfunction, lupus nephritis, nephrotoxic drugs, insulin therapy, malignancies, infectious diseases, and hospital admission in the recent months. The study protocol was approved by the local Ethics Committee of the Tehran University of Medical Sciences. Written informed consent was obtained from all participants prior to the study.

**Clinical and Laboratory Measurements**

All participants’ weight and height were measured, and their body mass index (BMI) was determined (kg/m²). Waist circumference (WC) was also measured (in centimeters). Venous blood samples were collected from each participant after a 12-h overnight fasting for biochemical analysis. Serum fasting blood sugar (FBS) and 2-h post-prandial (2HPP) glucose were determined by the glucose oxidase method based on Trinder [22]. For the assessment of glycated hemoglobin A1c (HbA1c), high-performance liquid chromatography was performed according to the method of Lahousen et al. [23]. Plasma lipid profile including serum total cholesterol [24], triglyceride, high-density lipoprotein cholesterol (HDL-C) [25], and low-density lipoprotein cholesterol (LDL-C) [26] were determined by using enzymatic method with commercially available kits (Pars Azmoon, Karaj, Iran). Measurement of serum Cr was performed by enzymatic method. Urinary albumin excretion was measured by calorimetric methods using commercial kits (ZiestChem Diagnostics, Tehran, Iran). NGAL and RBP-4 were both measured using Enzyme-Linked Immunosorbent Assay kit (Bioassay Technology, intra-assay CV < 8%, inter-assay CV < 10%). GFR was calculated using the CKD-EPI method [27]. Based on the KDIGO, CKD was defined as kidney damage or GFR < 60 mL/min/1.73 m² for 3 months or more, irrespective of cause.

**Statistical Analysis**

STATA software version 12 for Windows (Stata Corp., College Station, TX, USA) was used for statistical analysis. Continuous variables were demonstrated as mean ± SD. Categorical outcomes are introduced as proportions. Design-based parametric independent t test or nonparametric Mann-Whitney U Test was used to compare continuous data between the binary variables. Multiple Logistic regression models were used to examine the association of albuminuria with NGAL and RBP-4. This association was adjusted for age and sex in model 1 and for age, sex, WC, FBS, 2HPP, HbA1c, cholesterol, HDL, LDL, TG, and BMI in model 2. p value < 0.05 was considered statistically significant. We used model 2 to assess the association between GFR, NGAL, and RBP-4. Multiple linear regression models adjusted for duration of diabetes, FBS, HbA1c, HDL, LDL, total cholesterol, and BMI were used to examine the association of GFR with NGAL and RBP. The C-statistics was used to assess the discriminatory power of NGAL and RBP-4 for prediction of CKD in patients with type 2 diabetes.
Results

Basic Characteristics of Diabetic Patients and Control Group

One hundred and thirty-three patients including 72 females (40.7%) were enrolled in this study. Patients with diabetes were classified into 3 groups based on their ACR. Forty-four patients were counted for normal albuminuria group (26 females; mean age 51.81 ± 7.04), moderately increased albuminuria (microalbuminuria) group consisted of 45 patients (17 females; mean age 53.71 ± 9.40), and 44 patients were accounted for severely increased albuminuria (macroalbuminuria) group (20 females; mean age 58.16 ± 6.88). Other baseline characteristics of the patients enrolled in this study are presented in Table 1. Lipid profile showed no overall significant association with albuminuria, except for LDL-C, which was significantly different in patients with severely increased albuminuria as compared to the control group (97.89 ± 30.06, 116.5 ± 33.4, respectively, p value = 0.049). GFR was significantly lower among patients with severely increased albuminuria compared to the control group and those with normal and moderately increased albuminuria (94.01 ± 6.03 vs. 99.5 ± 11.02, 86.19 ± 12.93, 72.06 ± 11.69, respectively).

Univariate Correlation for NGAL and RBP-4 and Multiple Regression Models

Urine levels of NGAL and RBP-4 were significantly higher in all 3 groups of patients with diabetes compared to the control group (Table 1). In addition, patients with severely increased albuminuria had higher levels of NGAL and RBP-4 compared to the patients with normal albuminuria (129.13 ± 13.6 vs. 114.08 ± 16.38, 52.47 ± 8.00 vs. 43.64 ± 10.8, respectively), but there was no statistically significant difference between the severely increased albuminuria
and moderately increased albuminuria groups (129.13 ± 13.6 vs. 122.17 ± 13.25, 52.47 ± 8.00 vs. 49.58 ± 10.43, respectively; Table 1).

Two models were considered; model 1 was adjusted for age and sex and model 2 was adjusted for all confounding variables such as age, sex, WC, FBS, 2HPP, HbA1c, cholesterol, HDL, LDL, TG, and BMI as listed in Table 1. Both models showed a statistically significant difference of NGAL and RPB levels among the patients with diabetes compared to the control group and also among diabetics with moderately and severely increased albuminuria compared to diabetics with normal levels of urinary albumin (Table 2). However, when analyzing the difference of these 2 biomarkers between patients with moderately and severely increased albuminuria, the 2 models revealed different results. uNGAL levels were significantly higher among diabetic patients with severely increased albuminuria compared to diabetic patients with moderately increased albuminuria in the first model (50.16 vs. 43.99), while RBP-4 revealed the same significant trend in the second model (21.99 vs. 17.61; Table 2). Also, the result of logistic regression model, for CKD is provided in Table 3. Results from this study showed a positive correlation between NGAL, RBP-4, and CKD with regression coefficients of 0.50 (p value = 0.012) and 0.08 (p value = 0.002), respectively. As well as negative correlation among NGAL, RBP-4 and eGFR with regression coefficient of 0.2 (p value = 0.02) and 1.06 (p value = 0.05), respectively (Table 3).

### Table 2. Relation between urinary NGAL and RBP-4 and albuminuria

<table>
<thead>
<tr>
<th></th>
<th>NGAL</th>
<th>RBP-4</th>
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</thead>
<tbody>
<tr>
<td></td>
<td>model 1</td>
<td>model 2</td>
</tr>
<tr>
<td>Control</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Normal albuminuria</td>
<td>35.3 (&lt;0.001)</td>
<td>34.26 (&lt;0.001)</td>
</tr>
<tr>
<td>Moderately increased albuminuria</td>
<td>43.99 (&lt;0.001)</td>
<td>43.42 (&lt;0.001)</td>
</tr>
<tr>
<td>Severely increased albuminuria</td>
<td>50.16 (&lt;0.001)</td>
<td>48.38 (&lt;0.001)</td>
</tr>
<tr>
<td>Normal albuminuria</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Moderately increased albuminuria</td>
<td>8.59 (0.003)</td>
<td>9.17 (0.003)</td>
</tr>
<tr>
<td>Severely increased albuminuria</td>
<td>14.76 (&lt;0.001)</td>
<td>14.12 (&lt;0.001)</td>
</tr>
<tr>
<td>Moderately increased albuminuria</td>
<td>Reference</td>
<td>Reference</td>
</tr>
<tr>
<td>Severely increased albuminuria</td>
<td>6.17 (0.035)</td>
<td>4.95 (0.097)</td>
</tr>
</tbody>
</table>

Data are presented as β coefficient (p value).

Model 1: adjusted for age, sex; Model 2: adjusted for age, sex, WC, FBS, 2HPP, HbA1c, cholesterol, HDL, LDL, TG, BMI.

WC, waist circumference; FBS, fasting blood sugar; 2HPP, 2-h postprandial; HbA1c, hemoglobin A1c; HDL, high-density lipoprotein; LDL, low-density lipoprotein; TG, triglyceride; BMI, body mass index; NGAL, neutrophil gelatinase-associated lipocalin; RBP-4, retinol-binding protein-4.

### Table 3. Multiple logistic regression analysis determining the association between eGFR, DKD, and NGAL and RBP-4 in patients with type 2 diabetes

<table>
<thead>
<tr>
<th>Biomarkers</th>
<th>eGFR</th>
<th>DKD</th>
</tr>
</thead>
<tbody>
<tr>
<td>NGAL</td>
<td>–0.2 (–0.375 to –0.25), 0.02</td>
<td>0.05 (0.01 to 0.09), 0.012</td>
</tr>
<tr>
<td>RBP-4</td>
<td>–1.06 (–0.213 to –0.001), 0.05</td>
<td>0.08 (0.03 to 0.13), 0.002</td>
</tr>
</tbody>
</table>

Data are presented as coefficient (95% CI), p value.

NGAL, neutrophil gelatinase-associated lipocalin; RBP-4, retinol-binding protein-4; eGFR, estimated glomerular filtration rate; DKD, diabetic kidney disease.
Receiver Operating Characteristic Curve Analysis of NGAL and RBP-4

For determining the diagnostic index of NGAL and RBP-4 in early detection of DN in patients with normal urinary albumin level and eGFR, receiver operating characteristic curve analyses were performed. The sensitivity and specificity for NGAL was calculated as 90.4% and 49.2% respectively. The sensitivity and specificity for RBP-4 were calculated as 84.6% and 62.5%, respectively. The analysis showed that these 2 proteins are rather sensitive than specific. Area under the curve (AUC) for NGAL was calculated as 80.5% with a cutoff value of 109. AUC for RBP-4 was calculated as 74.6% with cutoff value of 46.1. According to our analysis, NGAL is good, and RBP-4 is a fair complementary diagnostic tool for early detection of DKD (Fig. 1).

Discussion

Early diagnosis and prevention of DN which is the leading cause of morbidity and mortality among patients with diabetes and accounts for enormous medical expenses worldwide is of utmost importance. Our study has determined the diagnostic profile of the urine level of NGAL and RBP-4 in early detection of advanced DKD even before changing glomerular markers such as increasing albuminuria and decreasing GFR levels. Although albuminuria and low GFR have
been the 2 most common markers for diagnosis of DN so far, there are several limitations to the measurement of albuminuria as a biomarker of DKD. First of all, albuminuria can be increased as a result of comorbidities of diabetes (e.g., obesity and hypertension), making it a less specific marker for diagnosis of DKD [28]. Second, albuminuria is not observed in 30–40% of patients with type 2 diabetes and kidney disease [29]. Third, hypertension is often treated with renin-angiotensin-aldosterone inhibitors which reduce the GFR, and thus albuminuria is normalized [30]. Tubular damage in 30% of type 2 diabetics with DN occurs in advance to glomerular damage [31]. This has prompted many researchers to look for and assess the diagnostic accuracy of surrogate markers for DKD. uNGAL and RBP-4 are 2 proteins that can be suggestive of tubular damage [32]. NGAL binds with iron forming NGAL:siderophore:Fe3b complex which is essential for nephron formation [33]. In addition, NGAL has significant kidney protective activities through increasing proliferative state and reducing apoptosis [34]. Synthesis of NGAL is upregulated in the distal and proximal tubule cells as a result of ischemia in response to kidney injury [35, 36]. This is the reason why NGAL is quickly increased in the urine in response to tubular injury. Also, RBP-4 is a specific carrier for retinol (vitamin A alcohol) in the blood that is produced by liver and fat cells [37]. Elevated urine level of this marker that is completely reabsorbed in the proximal tubule accounts for proximal tubular cells damage in patients with DKD [16]. There are several possible explanations for this increase. One of them could be that urinary RBP might reflect tubule-interstitial fibrosis since its excretion is directly related to the proximal tubular cells function. Another possible explanation is that elevated urinary RBP could be due to the impairment in one of the mechanisms that indirectly affects proximal tubular cells function (i.e., impairment in reabsorption machinery, glomerular filtration barrier, massive production of low molecular weight protein) [17]. Also, associations have been found between RBP and insulin resistance, which may be due to an impaired insulin signaling pathways in skeletal muscle [38].

Various studies have investigated the association between albuminuria and the level of NGAL and RBP-4 in the urine of patients with and without type 2 diabetes. Many previous studies indicate that urine NGAL and RBP-4 levels are higher in patients with diabetes once compared to control groups as well as they are positively correlated with albuminuria in diabetic patients [1, 8, 10, 12–16, 39, 40]. Moreover, urine NGAL and RBP-4 are significantly greater in diabetic patients with moderately increased albuminuria in comparison to patients with normal albuminuria and control groups and are positively correlated with urine ACR [1, 19, 20, 32, 41]. Also, Wu et al. [1] realized that both NGAL and RBP-4 were independently associated with albuminuria. Of notable mention, some studies found out that uNGAL and RBP-4 were higher in normoalbuminuric diabetics compared to control groups. This implies the possible role of urinary level of NGAL and RBP-4 as predictors for early detection of DN as it warns tubular damage which precedes moderately increased albuminuria via glomerular damage [13, 16, 32, 42]. Some studies defined that NGAL could be an indicative of not only the onset of DN but also the rate of DN progression [6, 43]. Also, RBP-4 could be used for follow-up of patients with DN to monitor the progression of their disease [16, 44].

Findings from this study showed statistically significant higher uNGAL and RBP-4 levels in patients with type 2 diabetes in comparison to the healthy control group. These 2 proteins were higher not only in albuminuric diabetic patients compared to nonalbuminuric diabetic patients but also in diabetic patients with severely increased albuminuria once compared to patients with moderately increased albuminuria.

Various studies have evaluated the association between uNGAL and GFR in patients with type 2 diabetes. The results from the studies on this issue are conflicting as Satirapoj [43] in a recent review article suggested that an increase in urinary level of NGAL is independent of a decrease in GFR. Also, Rotbain Curovic et al. [9] have recently shown that NGAL is not a predictor of GFR. Moreover, a few studies have attempted to assess the association between urinary RBP-4 levels and GFR that have determined the negative correlation between them.
in non-diabetic patients with CKD [17]. In addition, some other studies had similar results to ours, indicating a statistically significant negative correlation between both NGAL and RBP-4 levels with GFR [16, 18–21] (Table 3).

Previous studies similar to ours assessed diagnostic accuracy of the urine level of NGAL and RBP-4 as biomarkers of DKD in patients with type 2 diabetes. Most of them have presented AUC of 80% or above for both factors which was significant [11, 16, 19, 45]. Furthermore, our result showed that AUCs of NGAL and RBP-4 for the diagnosis of CKD in type 2 diabetic patients with DN were 87.5 and 88.7%, respectively. The AUC could determine valuable statistical comparison of diagnostic tests [46].

According to the positive correlation of urine levels of NGAL and RBP-4 with albuminuria and the high diagnostic accuracy of these 2 biomarkers for early detecting of CKD in type 2 diabetic patients, our findings imply that NGAL and RBP-4 could be used as complementary measurements to albuminuria in early diagnosis of CKD in patients with type 2 diabetes.

One of the strengths of this study that helped boost the discriminative strength of the markers and generalization of the findings is the inclusion of both diabetic and nondiabetic patients as well as classification of diabetic patients into 3 groups with normal albuminuria, moderately increased albuminuria, and severely increased albuminuria. We were able to analyze the difference of NGAL and RBP-4 between nondiabetics and diabetics as well as within the diabetic group. Moreover, this is one of the very few studies measuring both NGAL and RBP-4 simultaneously.

Cross-sectional nature of the study and relatively low sample size were limitations of this study. This study has a cross-sectional design. Whether the damage markers, as assessed in our study, predict the progression of DN has to be investigated in large, long-term, prospective, observational studies. Moreover, we have only measured the levels of NGAL and RBP-4 in the 24-h urine sample. Measurement of serum levels of these 2 proteins is suggested in the future studies to further explore the role of NGAL and RBP-4 in the diagnosis and prognosis of DN. Lastly, although potential confounders have been adjusted, residual confounding may exist due to measurement errors or unmeasured factors.

**Conclusion**

uNGAL and RBP-4 may serve as efficient biomarkers of tubular damage, and thus could potentially be used as complementary measurements to the conventional approaches for diagnosis of DN in patients with type 2 diabetes. Further long-term, prospective, observational studies should be designed to determine the association between these 2 markers and the progression of DN.

**Acknowledgments**

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**Statement of Ethics**

All procedures performed in studies involving human participants were in accordance with the ethical standards of the institutional and/or national research committee and with the 1964 Helsinki Declaration and its later amendments or comparable ethical standards. Informed consent was obtained from all individual participants included in the study.
Disclosure Statement

The authors declare that they have no competing interest.

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Author Contributions

A.E., M.N., and F.F.A.: conception or design of the work. F.M., S.M.F.A., and F.D.F: drafting the article. P.K., F.M., B.A., and M.A.: data analysis and interpretation. A.E.: critical revision of the article. All the authors approved the final version and have the agreement to be accountable for all aspects of the work in ensuring that questions related to the accuracy or integrity of any part of the work are appropriately investigated and resolved.

Data Availability

All data generated or analyzed during this study will be made available on request.

References