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ORIGINAL ARTICLE

Accuracy of Urinary Epidermal Growth Factor to Creatinine Ratio to Predict 24-Hour Urine Epidermal Growth Factor and Interstitial Kidney Fibrosis in Patients with IgA Nephropathy

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SUMMARY

Background: **Urinary levels of EGF may be a noninvasive biomarker of the degree of interstitial fibrosis. However, all the available data are based on studies that examined the EGF/creatinine ratio in spot urine samples. The agreement between EGF/creatinine ratio and 24-hours EGF excretion has not been analyzed, neither has it been established which of these two measurements is a better predictor of the degree of interstitial fibrosis. To investigate whether the EGF/creatinine ratio can predict 24-hours EGF, and which of these two measures is a better predictor of interstitial fibrosis in patients with IgA nephropathy (IgAN).**

Methods: **This is a cross-sectional study including 80 patients with IgAN. EGF levels were measured by ELISA in spot second-morning and 24-hours urine samples. We analyzed the concordance between these two measures and their respective ability to predict interstitial kidney fibrosis.**

Results: **The intraclass correlation coefficient between 24-hours and spot EGF/creatinine was 0.63 (95% CI: 0.54 - 0.70), bias was 2.7 µg/mL (95% CI: 2.1 - 7.5). Passing-Bablok regression did not show a significant deviation from linearity (p = 0.72). Bland-Altman showed a systematic and proportional error between both EGF measures. Spot EGF/creatinine ratios overestimated the 24-hours EGF at low excretion values and underestimated it at high excretion values. In univariate analyses, 24-hours excretion of EGF was a better predictor of interstitial fibrosis than spot EGF/creatinine ratio (R^2: 0.43 vs. 0.30, p = 0.000). In multivariate analyses, the 24-hours excretion of EGF plus GFR, significantly improved the prediction of interstitial fibrosis when compared with GFR alone (R^2: 0.52 vs. 0.39, p = 0.000). When spot-urine EGF was introduced instead of the 24-hours excretion, the model was statistically significant but had a lower predictive capacity (R^2: 0.46 spot EGF/creatinine vs. R^2: 0.52 24-hours EGF excretion, p = 0.000).**

Conclusions: **The 24-hours excretion of EGF should be considered as the first-choice measure to estimate the interstitial fibrosis. The EGF/creatinine ratio cannot accurately estimate the total EGF excretion of but it also improves the estimation of the fibrosis surface, and, consequently, could be an alternative whenever 24-hours urine samples cannot be obtained.**

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KEY WORDS

IgA nephropathy (IgAN), epidermal growth factor (EGF), spot EGF/creatinine ratio, 24-hours EGF excretion, kidney fibrosis, kidney interstitial fibrosis biomarkers

INTRODUCTION

IgA nephropathy (IgAN) is one of the most common glomerular diseases and leads to chronic renal failure in 25 to 30% of patients in the long-term [1]. Besides proteinuria and hypertension, the glomerular filtration rate (GFR), and the degree of kidney interstitial fibrosis surface at diagnosis are considered to be the best surrogate prognostic measures [2-4]. Since the effective filtration area is usually reduced in parallel with the increase in the surface of interstitial fibrosis and tubular atrophy, it is assumed that the measurement of GFR indirectly reflects the degree of interstitial fibrosis [5]. However, in the early stages of glomerular diseases, due to the hyperfiltration of the remaining nephrons, a progression of the glomerular and tubulo-interstitial lesions can occur, without significant changes in tGFR [6]. On the other hand, in IgAN, the severity of interstitial fibrosis lesions does not show a homogeneous distribution but can vary focally along the renal parenchyma. Thus, kidney biopsy sampling is subject to a random error that can result in discordant data between the degree of fibrosis observed in the biopsy samples and the actual degree of fibrosis of the whole renal parenchyma. These reasons justify the need of studies to identify early-stage noninvasive predictors of interstitial fibrosis and tubular atrophy. The value of urinary excretion of epidermal growth factor (EGF) as an early predictor of kidney fibrosis in IgA nephropathy has been analyzed in several studies [7-10]. EGF is produced by the ascending portion of Henle's loop and the distal convoluted tubule. It is considered to be a trophic factor for renal tubular cells [11]. Urinary levels of EGF are not influenced by the filtration of circulating EGF, but reflect the local production by renal tubular cells [12,13]. A significant correlation between urinary excretion and renal expression of EGF has been demonstrated [7,10], and a significant correlation between urinary levels of EGF and the severity of interstitial fibrosis and tubular atrophy has been shown [7,9,10]. Three independent studies have shown that the measurement of urinary EGF excretion could be of prognostic value in IgA nephropathy [7-9]. Moreover, two recent studies highlight the potential prognostic value of urinary EGF, identifying it as an independent predictor of chronic kidney disease progression [10,14]. In a previous pilot study, we confirmed that the EGF/creatinine ratio in spot urine samples, in association with either interleukin-6 or monocyte attractant protein type-1 levels, significantly improved the estimation of the surface of interstitial fibrosis in patients with IgA nephropathy [15]. Overall, these data indicate that urinary levels of EGF may be a potential surrogate marker of the degree of interstitial fibrosis [16]. However, all the clinical data currently available on the clinical value of urinary EGF are based on studies that examined the EGF/creatinine ratio, measured in spot urine samples. The agreement between EGF measurements obtained from spot urine samples and those obtained from 24-hours excretion has not yet been evaluated, nor has the association been compared between each of these measurements and the extent of interstitial fibrosis. In this study, we analyzed the accuracy of the spot urine EGF/creatinine ratio to predict 24-hours EGF excretion, and we compared the ability of each measurement to predict the degree of interstitial fibrosis, in a cohort of patients with IgAN.

MATERIALS AND METHODS

Patients and study design

The study included 80 consecutive patients with primary IgAN confirmed by renal biopsy with > 10 glomeruli and GFR ≥ 30 mL/min/1.73 m^2. We collected 24-hours and spot second-morning urine samples from each patient. 24-hours urine samples were collected the day before kidney biopsy (from 9 am to 9 am). Spot second-morning urine samples were obtained the day of kidney biopsy (before kidney biopsy). At the time of urine sample collection, no patient had received treatment with angiotensin II receptor blockers, Angiotensin-Converting Enzyme inhibitors, angiotensin II receptor blockers associations, aldosterone antagonists, cimetidine, cotrimoxazole, or any other drug that alters tubular creatinine secretion. In addition, none of the patients had been treated with steroids, other immunosuppressive drugs, or paricalcitol, prior to inclusion in this study.

For standard biochemical measures, blood was obtained from the antecubital vein and put into glass tubes containing no additives. Urine samples were centrifuged at 1,500 x g for 10 minutes and stored at -80°C until processing. The presence of pyuria was discarded by a study of the urinary sediment. By comparing the total creatinine in the sample with the predicted creatinine, we assessed the adequacy of the 24 hours collection using the following formulas: 28 - (0.2 x age) x kg in men and 23.8 - (0.17 x age) x kg in women in mg/day [17]. 24-hours urine samples with creatinine excretion values lower than the mean - 2SD of the expected values for age and GFR, were discarded and recollected within the next two weeks after kidney biopsy.

Biochemical procedures

GFR was measured by Cr EDTA clearance within the 5 days following kidney biopsy [18]. Creatinine measurements were performed using a compensated isotope-dilution mass spectrometry (IDMS) - traceable method (Hitachi Modular P-800; Roche Diagnostics, Berlin, Germany). Urinary EGF was measured using a

commercial ELISA kit (Human EGF Quantikine ELISA Kit, R&D Systems, Minneapolis, MN, USA) following the manufacturer's instructions. All assays were performed in duplicate and calibrated with purified standards and reference serums obtained from the manufacturers. Intra-assay precision was obtained by assaying two patient samples with known concentrations 10 times on the same plate and was 5.9% at low (mean: 12.5 pg/mL) and 7.4% at high concentrations (mean: 150 pg/mL). Inter-assay precision was obtained by assaying two patient samples with known concentration in 10 separate assays and was 6.8% at low (mean: 13.2 pg/mL) and 7.1% at high concentrations (mean: 147 pg/mL). In spot-urine samples, EGF concentrations were adjusted for creatinine excretion (uCreat) and expressed as µg/g. 24-hours EGF excretion was expressed as µg/24 hours.

Kidney biopsies

The extent of interstitial fibrosis was analyzed by 3 trained nephropathologists on 5 µm Masson's trichrome stained slides by quantitative morphometry with an autoanalyzer (Olympus WCE2). The intraclass correlation coefficient for the quantification of the extent of interstitial kidney fibrosis among the three pathologists was 0.87 (95% CI: 0.70 - 0.96). For statistical analyses, we included the mean of these three measurements.

Statistical analysis

The results were expressed as mean ± standard deviation for normally distributed variables or as median and quartiles for non-normally distributed variables. Comparison of proportions was performed using the Chi-square test or the Fisher's exact test. The association between quantitative variables was analyzed using the Pearson`s correlation coefficient. The relationship between the values of spot EGF/creatinine and 24-hours EGF excretion was analyzed by the intraclass correlation coefficient and by the Passing-Bablok regression analysis. Bias was defined as the mean of individual differences between 24-hours EGF and spot EGF values. Precision was established as the standard deviation of bias [19]. The Student's *t*-test for paired samples was used to assess differences between 24-hours EGF and spot EGF/creatinine ratios values. The limits of agreement between the values measured in the two samples were analyzed by the Bland-Altman method [20]. Comparisons among more than two means were done with analysis of variance. To analyze the potential role of spot and 24-hours EGF excretion to predict the extent of interstitial fibrosis, a univariate analysis was performed, followed by a stepwise multiple regression analysis, taking the degree of interstitial fibrosis as the dependent variable, after logarithmic transformation and verification of the normality of its distribution. In all cases, a p-value < 0.05 was considered statistically significant. SPSS version 20.0 (IBM Corp, IBM SPSS Statistics for Windows; Armonk, NY, USA) and Med-Calc (Mariakerke, Belgium) statistical software packages were used for the analyses.

RESULTS

The clinical, biochemical, and histopathological characteristics of the study cohort are summarized in Table 1.

Precision and bias of the spot EGF/creatinine ratio in predicting 24-hours EGF excretion

In the whole group, the intraclass correlation coefficient between 24-hours and spot EGF measurements was 0.63 (95% CI: 0.54 - 0.70) and bias was 2.7 (95% CI: -2.1 - 7.5 µg/mL). Figure 1 shows the results of the Passing-Bablok regression analysis between spot and 24-hours measurements of EGF. The intercept of the regression equation was -19.74 (95% CI: -26.08 - (-13.39)). The slope was 2.00 (95% CI: 1.69 - 2.32). The regression model did not show significant deviation from linearity ($p = 0.72$). Figure 2 shows the Bland-Altman graph analyzing the agreement between 24-hours EGF and spot EGF/creatinine ratio and the limits of agreement between them. The distribution of points indicates a proportional systematic bias between the two measurements. According to the results obtained using the Passing-Bablok and Bland-Altman models, the relationship between the two measurements did not meet the interchangeability criteria and indicated that there was a systematic and proportional bias between spot and 24-hours measurements. Spot EGF/creatinine ratios overestimated the 24-hours EGF at low excretion values and underestimated it at high excretion values.

Prediction of interstitial fibrosis

In single regression analyses, interstitial fibrosis was associated with age, GFR, proteinuria, spot-EGF, and 24-hours EGF excretion. Among them, 24-hours EGF excretion was the best single predictor (Table 2). In multiple regression analysis, GFR and 24-hours EGF were found to be the only independent predictors of interstitial fibrosis and, together, accounted for 52% of its variability. The addition of the 24-hours excretion of EGF to the model including GFR significantly improved the prediction of the surface of interstitial fibrosis (R^2: 0.52 vs. 0.39, $p = 0.000$). When spot-urine EGF was introduced instead of the 24-hours excretion, the model maintained statistical significance but had a significantly lower predictive capacity (R^2: 0.46 spot EGF/creatinine vs. R^2: 0.52 24-hours EGF excretion, $p = 0.000$).

DISCUSSION

Our study provides two relevant results. First, it indicates that the spot urine EGF/creatinine ratio does not provide an accurate estimation of 24-hours EGF. The EGF/creatinine ratio and the 24-hours urinary excretion of EGF did not meet the interchangeability criteria and there was a systematic and proportional bias between

Table 1. Clinical, biochemical, and histopathological characteristics of the study cohort (n = 80).

Age (years)	**49.3 ± 24.5**
Gender (m/f)	**55 (68.9)/25 (31)**
Creat (mg/dL)	**1.1 ± 0.9**
GFR (mL/min/1.73 m^2)	**85.9 ± 16.5**
Prot (g/24 h)	**1.79 ± 0.61**
Hem (cell/µL)	**99 ± 45**
SBP (mmHg)	**138 ± 18**
DBP (mmHg)	**76 ± 19**
24-hours EGF excretion (µg/24h)	**21.4 ± 11.9**
Oxford classification	
M1	**21 (26)**
E1	**23 (28.7)**
S1	**23 (28.7)**
To	**40 (50)**
T1	**22 (27.5)**
T2	**18 (22.4)**

SBP - systolic blood pressure, DBP - diastolic blood pressure, EGF - epidermal growth factor, GFR - glomerular filtration rate, Prot: 24-hours proteinuria.
Qualitative data are expressed as absolute frequency (percentage).
Quantitative data are represented by the mean ± SD.

Table 2. Variables associated with the surface of interstitial kidney fibrosis in the univariate (a) and in the multivariate (b) analyses.

Variables	β	t	Sig	R^2
Univariate				
Age (years)	**0.36**	**5.6**	**0.002**	**0.19**
GFR (mL/min/1.73 m^2)	**-0.67**	**-12.9**	**0.000**	**0.39**
24-hours EGF (µg/24h)	**-0.76**	**-15.3**	**0.000**	**0.43**
Spot EGF (µg/g)	**-0.54**	**-9.1**	**0.000**	**0.30**
Proteinuria g/g	**0.24**	**3.5**	**0.013**	**0.15**

β - standarized coefficients.

them. Second, even though both measurements correlated with interstitial fibrosis and tubular atrophy, our results indicate that 24-hours EGF excretion was a more accurate predictor of interstitial fibrosis than the spot EGF/creatinine ratio, after adjusting for GFR. The assessment of the urinary excretion of molecules reflecting glomerular and/or tubular damage is a keystone for the clinical evaluation of glomerular diseases. The 24-hours urine collections used to measure the total daily excretion can be cumbersome and unreliable because of over- or under-collection. To overcome these disadvantages, spot urine/creatinine ratios are commonly used to estimate the bulk of daily excretion. These ratios are generally adequate for variables such as proteinuria or microalbuminuria, which do not depend directly on the amount of total tubular cells [21]. However, when used to estimate the 24-hours EGF excretion, the EGF/creatinine ratio could introduce new sources of error, since the levels of both molecules (EGF and creatinine), decrease as renal function declines and the number of renal tubular cells decrease. Therefore, the significance of this ratio depends on the relative reduction of the levels of each of the two molecules involved in its calculation. A proportional reduction will lead to the ratio remaining unaltered despite the progressive reduction of the number of tubular cells. Conversely, if the decrease is not

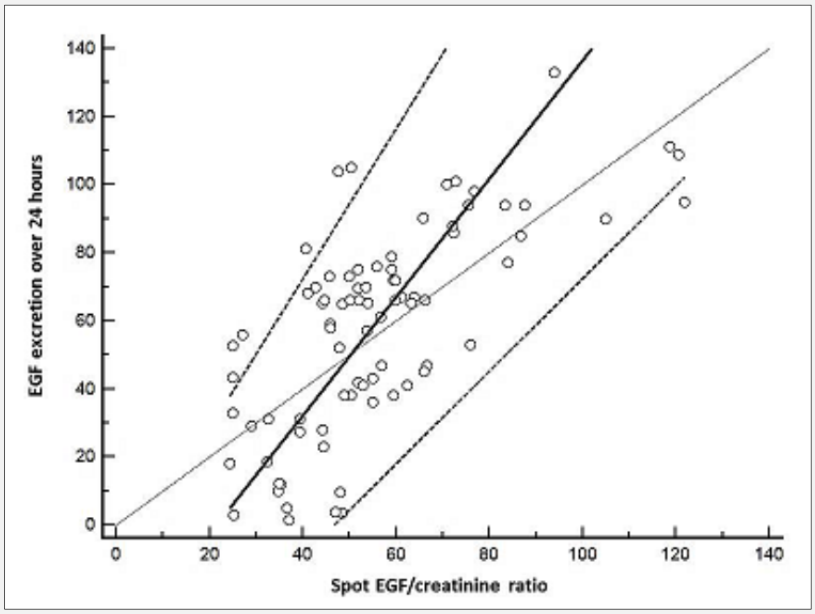

Figure 1. Passing & Bablok regression analysis between 24-h EGF and measurements spot/creatinine ratio.

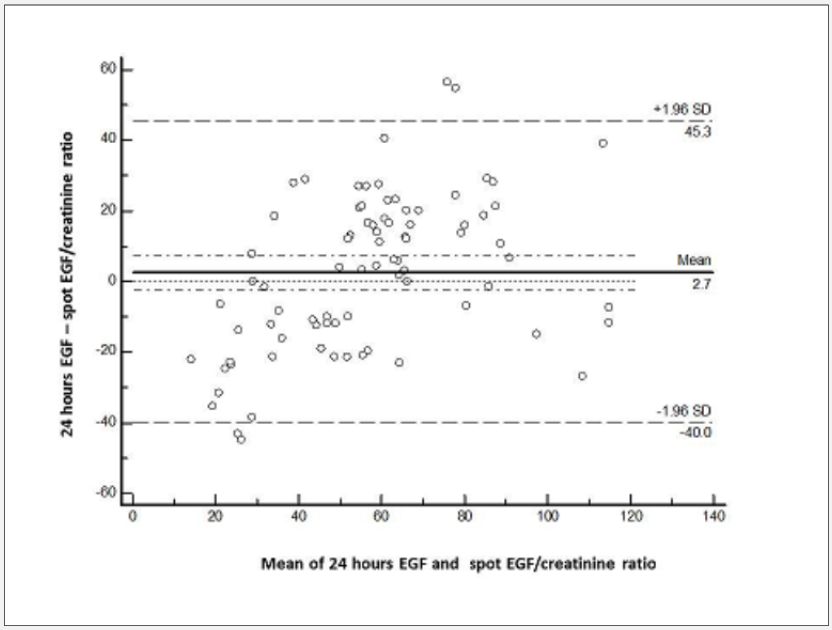

Figure 2. Bland-Altman graph analyzing the agreement between 24-h EGF excretion and spot EGF/creatinine ratio.

proportional, the ratio could under- or overestimate the total excretion of EGF. As previously mentioned, evidence has been reported that urinary EGF/creatinine ratio can be considered as a noninvasive biomarker of tubular atrophy and fibrosis [7,9-12]. Our data are in concordance with these results but indicate that the 24-hours excretion of EGF is a better predictor of interstitial fibrosis than the spot-EGF/creatinine values. None of the studies performed previously, measured the 24-hours EGF excretion. Therefore, it is not possible to assess whether, as observed in our patient cohort, in those studies interstitial fibrosis could have been more accurately predicted by the total excretion of EGF than by the EGF/creatinine ratio. Our data support that the measurement of 24-hours excretion of EGF has the potential to become a useful noninvasive biomarker of interstitial kidney fibrosis for clinicians. GFR is so far the best available measure of renal function. However, when GFR is measured by an objective isotopic method, using molecules that are filtered but not processed by the tubules, it gives a measure of the glomerular filtration area which does not always run in parallel with the amount of tubular cells, whereas when estimated by means of equations based on creatinine, GFR is subject to the influence of variables unrelated to renal function such as gender, diet, muscle mass or pharmacological interferences [22]. The urinary excretion of EGF varies proportionally to the number of tubular cells and improves the prediction of the interstitial fibrosis surface when added to GFR. This information can be especially useful to assess tubular injury in the early phases of disease, in which, due to the compensatory hyperfiltration of the remaining nephrons, the progression of tubular and interstitial lesions does not translate into a reduction of GFR.

The main strengths of our study are the measurement of glomerular filtration through a direct and objective method and the inclusion of patients with a wide range of GFRs. The main limitation of our study is the absence of external validation.

CONCLUSION

In patients with IgA nephropathy, the urinary excretion of EGF significantly improves the prediction of the interstitial fibrosis surface provided by the GFR alone. The 24-hours excretion of EGF should be considered as the first-choice measure to predict the interstitial fibrosis surface. The EGF/creatinine ratio in spot urine samples cannot adequately estimate the total excretion of EGF and has a lower association with the interstitial fibrosis surface than the 24-hours EGF excretion. However, spot EGF/creatinine values also improve the estimation of the fibrosis surface, when compared to that provided by the measurement of the GFR alone and, consequently, could be an alternative whenever 24-hours urine samples cannot be obtained.

Statement of Ethics:

The local Ethics Review Board for human studies approved the study, and all patients provided informed consent.

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Author contributions:

Alfonso Segarra, Marisa Martin, and Carme Perich designed the study, performed the statistical analyses and wrote the final version of the manuscript.

Clara Carnicer and Cristina Martinez performed the biochemical procedures, wrote the methods section and critically reviewed the final version of the manuscript.

Elias Jatem and Maria Molina selected and recruited the patients, contributed to the discussion, and critically reviewed the final version of the manuscript.

Declaration of Interest:

The authors have no conflicts of interest to declare.

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