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The ratio of epidermal growth factor to monocyte chemotactic peptide-1 in the urine predicts renal prognosis in IgA nephropathy

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The production of cytokines by resident and non-resident renal cells during immunoglobulin A nephropathy (IgAN) plays a key role in the progression of renal damage. The aim of this study was to determine if measurements of urinary epidermal growth factor (EGF) and monocyte chemotactic peptide-1 (MCP-1), at the time of renal biopsy, were a predictor of end-stage renal disease (ESRD) in a cohort of 132 patients with biopsy-proven IgAN. Outcome measures were a doubling of the baseline serum creatinine (sCr) and/or ESRD. Patients with ratios of EGF/MCP-1 in the lowest tertile had a significant decline in renal survival, while patients in the highest tertile maintained 100% renal survival at 48 and 84 months of follow-up. Multivariate Cox's regression analysis showed that the urine EGF/MCP-1 ratio was an independent prognostic factor and indirectly correlated with the combined outcome. The predictive value was also measured by the area under the receiver operating characteristic curve (ROC). The area of the EGF/MCP-1 ratio was significantly higher than that of EGF or MCP-1 alone, histologic grade, creatinine clearance, or proteinuria. Our study suggests that the urinary EGF/MCP-1 ratio may be used as a prognostic marker of ESRD for patients with IgAN.

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Immunoglobulin A nephropathy (IgAN) is an immunocomplex-mediated glomerulonephritis characterized by the presence of IgA deposits in the mesangial area.[1] It is the most common primary glomerulonephritis (25–50% of diagnosed renal biopsies), and although initially thought to represent a benign condition, it is now recognized that IgAN leads to end-stage renal disease (ESRD) in a substantial proportion of patients within 10–20 years from the time of renal biopsy.[2] The clinical course is extremely variable ranging from persistent asymptomatic microscopic hematuria to rapidly progressive renal failure. The severity of histologic lesions, the presence of impaired renal function, arterial hypertension, and the degree of proteinuria at the time of renal biopsy are all well-known prognostic factors for the development of ESRD in IgAN patients.[3–6]

Progressive renal disease, regardless of the initiating insult, is characterized by glomerulosclerosis, tubulointerstitial fibrosis, and vascular sclerosis. The importance of the tubulointerstitial compartment in the progression of renal damage is supported by the evidence that the severity of tubulointerstitial changes, including tubular atrophy, interstitial cell infiltration, and fibrosis, is significantly correlated with the decline in renal function.[7]

There is an increasing body of evidence that the chemokine monocyte chemotactic peptide-1 (MCP-1), which promotes monocyte-specific chemotaxis, plays a major role in the progression of renal disease, both in animal models of renal damage and in different types of human renal disease.[8] Protein overload of proximal tubular epithelial cells results, *in vitro* and *in vivo*, in the increased tubular expression of MCP-1, which promotes the recruitment of inflammatory cells into the interstitium.[9,10] The extent of macrophage infiltration in the peritubular space is strongly correlated with the development of progressive interstitial fibrosis. Indeed, infiltrating macrophages represent both a source and a reservoir of profibrotic factors, including transforming growth factor-β (TGF-β), endothelin-1, and tumor necrosis factor-α (TNF-α), which induce matrix synthesis by resident

parenchymal cells. They can also release inhibitors of matrix-degrading proteases, such as tissue inhibitor of metalloproteinase-1 and plasminogen activator inhibitor-1.[11] Our group has previously shown that MCP-1 urine excretion is significantly correlated with its renal expression as well as with the extent of interstitial inflammatory infiltrate in several renal diseases.[12] On the other hand, epidermal growth factor (EGF), a 53-amino-acid peptide, produced by the ascending portion of Henle's loop and by the distal convoluted tubule, seems to modulate tissue response to injury in kidneys with tubulointerstitial damage. In this setting, we reported a decrease in the renal expression and urinary excretion of EGF.[13] On this basis, EGF urinary excretion has been suggested as a marker of tubular trophism. Our group demonstrated that the urinary levels of EGF inversely correlated with the extent of tubulointerstitial damage in IgAN.[14]

The aim of our study was to evaluate the prognostic value of the urinary excretion of EGF and MCP-1 ratio, at the time of renal biopsy, in predicting the progression of renal disease in a cohort of IgAN patients.

RESULTS

A cohort of 132 consecutive subjects was diagnosed as IgAN (91 men and 41 women). The general clinical characteristics of all patients are listed in Table 1. Mean age at renal biopsy was 31.6 ± 11.4 years. Microhematuria at onset was present in 63/132 (48%) and recurrent macrohematuria (MH) in 69/132 (52%) patients. Out of 132 patients, 48 (36.4%) patients were hypertensive at renal biopsy. At baseline, mean serum creatinine (sCr) was 1.2 ± 0.7 mg per 100 ml, estimated creatinine clearance (eCrCl) 95.3 ± 29.5 ml min^{-1}, and mean proteinuria (uPr) was 1.3 ± 1.1 g day^{-1}. Renal biopsy showed mild lesions (grades I–II) in 29/132 (22%) patients, moderate lesions (grade III) in 78/132 (59%), and severe lesions (grades IV–V) in 25/132 (19%). Ninety-eight patients, 74% of the total cohort, were treated with angiotensin converting enzyme inhibitors and/or angiotensin II receptor blockers in any period of the follow-up.

Urinary cytokine measurement

We evaluated EGF, MCP-1, and their ratio in the urine of all IgAN patients at the time of renal biopsy. The urinary levels of EGF, MCP-1, and EGF/MCP-1 ratio expressed as ng/mg uCr were 18.3 (8.03–44.5), 0.42 (0.2–0.7), and 48.9 (21.1–96.1), respectively.

Urinary EGF/MCP-1 ratio was significantly lower in 25 IgAN patients with severe histologic lesions compared to 78 subjects with moderate and 29 with mild histologic lesions (median EGF/MCP-1 (25th–75th percentile): severe, 8.9 (3.9–30.2); moderate, 43.0 (23.7–81.5); mild, 98.2 (54.6–152.2); severe vs moderate and moderate vs mild, $P = 0.001$; severe vs mild, $P < 0.0001$) (Figure 1).

There was a significant direct correlation between EGF/MCP-1 ratio and eCrCl ($R = 0.39$, $P < 0.0001$) and a significant inverse correlation between EGF/MCP-1 ratio and uPr ($R = 0.35$, $P < 0.0001$). The EGF levels correlated significantly with eCrCl ($R = 0.41$, $P < 0.0001$) and not with uPr ($R = 0.08$, $P = 0.38$), conversely, the MCP-1 levels correlated significantly with uPr ($R = 0.39$, $P < 0.0001$) and not with eCrCl ($R = 0.05$, $P = 0.58$).

The EGF/MCP-1 ratios corresponding to the first, second and third tertiles were 29.2, 70.4, 366.6, respectively. Baseline mean age, gender, proportion of patients with macrohematuria, and follow-up duration did not show statistically

Table 1 | Demographic and clinical characteristics of 132 IgAN patients

No. of patients	132
Gender (male)	91 (69%)
Age at renal biopsy (years)	31.6 ± 11.4
Macrohematuria (yes)	69 (52%)
Hypertension (yes)	48 (36%)
Serum creatinine (mg per 100 ml)	1.2 ± 0.7
Estimated creatinine clearance (ml min^{-1})	95.3 ± 29.5
Proteinuria (g per 24 h)	1.3 ± 1.1
EGF (ng/mg uCr)	18.35 (8.03–44.5)
MCP-1 (ng/mg uCr)	0.42 (0.22–0.74)
EGF/MCP-1	48.9 (21.1–96.1)
Renal lesions	
Mild (grades I–II)	29 (22%)
Moderate (grade III)	78 (59%)
Severe (grades IV−V)	25 (19%)
Therapy with ACE-I and/or ARB	98 (74%)
Follow-up (months)	54 (35–84)

ACE-I, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers; EGF, epidermal growth factor; IgAN, immunoglobulin A nephropathy; MCP-1, monocyte chemotactic peptide-1.
Data are expressed as mean ± s.d. or median and IQR, absolute frequency or percentage.

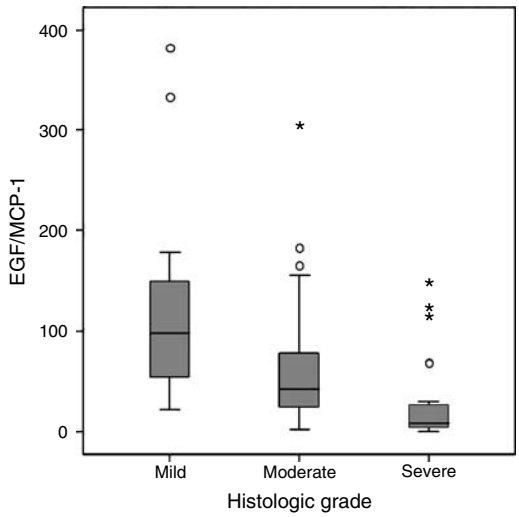

Figure 1 | Histologic grade and urinary values of EGF/MCP-1 ratio. The EGF/MCP-1 ratio was significantly different comparing mild vs moderate ($P = 0.001$) and severe ($P < 0.0001$) histologic lesions and comparing moderate vs severe ($P < 0.001$) histologic lesions (line crossing boxes show the median values; boxes show the interquartile range; whiskers show the largest and smallest observed values that are less than 1.5 box lengths from the 25th and 75th percentile; and the outlier represents a case with a value between 1.5 and 3 box lengths from the upper and lower edge of the box).

significant differences in the three groups. Instead, sCr, uPr, eCrCl, histologic grade, and the prevalence of arterial hypertension at renal biopsy showed statistically significant differences among the three tertiles. There were no statistically significant differences in the proportion of patients treated with angiotensin converting enzyme inhibitors and/or angiotensin II receptor blockers in the three groups (Table 2).

Renal survival

Twenty-seven events of the combined outcome occurred in a median follow-up of 54 (35–84) months from the renal biopsy, 24 in the first, 3 in the second, and none in the third tertile, respectively. Patients with the lowest EGF/MCP-1 tertile showed a renal survival of 73 and 36%, at 48 and 84 months of follow-up, respectively. Patients within the second tertile showed a renal survival of 94% at 48 and 84 months of follow-up, and those in the highest EGF/MCP-1 tertile showed a renal survival of 100% during the whole follow-up period (log-rank test, $P<0.0001$) (Figure 2).

Univariate analysis showed that baseline eCrCl, uPr, histologic grade, hypertension, EGF, and EGF/MCP-1 ratio were significantly associated with the outcome. MCP-1 showed only a weak association with the outcome, while no association was demonstrated for gender and age at renal biopsy. Unadjusted risk estimates of different risk factors for the combined outcomes are reported in Tables 3 and 4. Multivariate Cox's regression analysis showed that EGF, MCP-1, and EGF/MCP-1 ratio were independently associated with the combined outcome, after the adjustment for age and gender (data not shown), but also after the adjustment for eCrCl, uPr, histologic grade, and hypertension at renal biopsy (model 2) (Table 4). No interaction between the exposure variables and histologic grade, uPr, and/or eCrCl was found; the test for interaction did not modify the risk estimate of the outcome.

The ability of the EGF/MCP-1 ratio to predict the combined outcome was investigated by means of a receiver operating characteristic curve (ROC) analysis. To support the usefulness of EGF/MCP-1 ratio (rather than the two single measures), we performed an ROC analysis in which EGF, MCP-1, and EGF/MCP-1 ratio were compared. The areas under the curve (AUCs) (95% confidence interval (CI)) were 0.83 (0.76–0.89), 0.57 (0.49–0.66), and 0.91 (0.85–0.96), respectively. The differences between the areas were statistically significant between EGF and the ratio ($P=0.01$) and between MCP-1 and the ratio ($P<0.001$), demonstrating a better prediction capacity for the ratio (data shown in Figure 3a). Then, we compared our biomarker with the other covariates at baseline; the EGF/MCP-1 ratio showed an AUC and a 95% CI of 0.91 (0.84–0.95), compared to 0.76 (0.65–0.88) of eCrCl, 0.75 (0.63–0.88) of histologic grade,

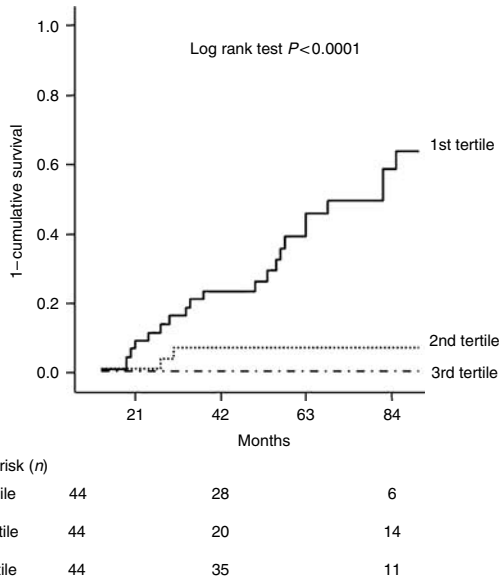

Figure 2 | Kaplan–Meier analysis of renal survival in the total cohort of patients divided into the tertiles of EGF/MCP-1 ratio. The number of events was significantly higher in the first tertile (log-rank test, $\chi^2=43.1$, $P=0.0001$). The combined outcome was ESRD and/or doubling of sCr. Patients at risk were the number of cases in observation at each time.

Table 2 | Demographic and clinical characteristics of 132 IgAN patients divided into three tertile groups of EGF/MCP-1 levels

Characteristics	Tertiles			*P*-value
	First	Second	Third	
No. of patients	44	44	44	
Age (years)	32.5 (11.2)	32.3 (11.8)	30.0 (11.4)	0.49
Gender (male)	33 (75%)	30 (68%)	28 (64%)	0.51
Macrohematuria (yes)	26 (59%)	22 (50%)	21 (48%)	0.52
Hypertension (yes)	22 (50%)	16 (36%)	10 (23%)	0.03
Serum creatinine (mg per 100 ml)	1.6 (0.9)	1.0 (0.2)	0.9 (0.2)	<0.0001
Urinary protein excretion (mg day^{-1})	1.8 (1.3)	1.1 (0.9)	1.0 (0.8)	0.003
Estimated creatinine clearance (ml min^{-1})	75.1 (30.2)	102.2 (23.8)	109.1 (23.0)	<0.0001
Histologic grade (mild/moderate/severe)	(1/25/18)	(10/31/3)	(18/22/4)	<0.0001
Therapy with ACE-I and/or ARB (yes)	34 (77%)	30 (68%)	34 (79%)	0.35
Follow-up duration (months)	58.3 (46.2)	51.0 (32.2)	59.6 (32.3)	0.11

ACE-I, angiotensin converting enzyme inhibitors; ANOVA, analysis of variance; ARB, angiotensin II receptor blockers; EGF, epidermal growth factor; IgAN, immunoglobulin A nephropathy; MCP-1, monocyte chemotactic peptide-1.
Data are expressed as mean ± s.d., absolute or percentage frequency; comparison between groups was made by one-way ANOVA, unpaired Student's *t*-test, or χ^2 test.

Table 3 | Unadjusted risk estimates by Cox's proportional hazard models for the combined outcome in 132 IgAN patients

Risk factor	Unit of increase or reference category	Unadjusted risk (HR) (95% CI)	*P*-value
Estimated creatinine clearance (ml min^{-1})	1	0.97 (0.96–0.98)	<0.0001
Histologic lesions grade	Mild	6.50 (3.26–12.96)	<0.0001
Proteinuria at renal biopsy (g per 24 h)	1	1.68 (1.36–2.08)	<0.0001
Hypertension at renal biopsy	0=no; 1=yes	0.31 (0.14–0.67)	0.003
Gender	0=male; 1=female	0.50 (0.19–1.34)	0.17
Age (years)	1	1.01 (0.98–1.04)	0.50
Onset type	0=mH; 1=MH	1.30 (0.60–2.82)	0.50
Therapy with ACE-I and/or ARB	0=no; 1=yes	0.76 (0.32–1.81)	0.54

ACE-I, angiotensin converting enzyme inhibitors; ARB, angiotensin II receptor blockers; CI, confidence interval; EGF, epidermal growth factor; HR, hazard ratio; IgAN, immunoglobulin A nephropathy; MCP-1, monocyte chemotactic peptide-1; mH, microhematuria; MH, macrohematuria.
Data are expressed as unadjusted HR and 95% CIs.

Table 4 | EGF, MCP-1, and the EGF/MCP-1 ratio risk estimates by Cox's proportional hazard models for the combined outcome in 132 IgAN patients

	EGF/MCP-1 ratio		EGF (ng/mg uCr)		MCP-1 (ng/mg uCr)	
Models	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value	HR (95% CI)	*P*-value
Unadjusted (1)	0.93 (0.90–0.96)	<0.0001	0.95 (0.92–0.98)	0.002	1.25 (0.98–1.59)	0.067
Adjusted (2)	0.94 (0.91–0.98)	0.002	0.95 (0.92–0.98)	0.002	1.35 (1.04–1.80)	0.024

CI, confidence interval; eCrCl; estimated creatinine clearance; EGF, epidermal growth factor; HR, hazard ratio; IgAN, immunoglobulin A nephropathy; MCP-1, monocyte chemotactic peptide-1; uPr, mean proteinuria.
Model 1 (unadjusted HR); model 2 (adjusted for eCrCl, uPr, hypertension, and histologic grade).

and 0.68 (0.56–0.81) of uPr. EGF/MCP-1 ratio reached the highest sensitivity and specificity in the prediction of the combined outcome (Figure 3b). The cutoff values for EGF/MCP-1 ratio, eCrCl, and uPr were 23.2 (sensitivity 88.9%; specificity 86.4%), 88.8 ml min^{-1} (sensitivity 76.9%; specificity 71.1%), and 0.71 g day^{-1} (sensitivity 77.8%; specificity 61.0%), respectively. Moreover, we explored the differences in the AUCs between EGF/MCP-1 ratio and all the other risk factors, and found statistically significant results between EGF/MCP-1 ratio and eCrCl ($P=0.002$), EGF/MCP-1 ratio and uPr ($P=0.004$), and between EGF/MCP-1 ratio and histologic grade ($P=0.005$). No statistically significant results were demonstrated between eCrCl and uPr, eCrCl and histologic grade, and uPr and histologic grade.

Finally, to confirm our results of survival analysis, we explored the association between EGF/MCP-1 ratio and the rate of loss of eGFR. First, we correlated EGF/MCP-1 ratio with eCrCl slope, demonstrating a statistically significant direct correlation ($R=0.24$, $P=0.0091$). Then, we performed a multiple regression analysis, adjusting for the other covariates at baseline (eCrCl, uPr, and histologic grade), also demonstrating in this further analysis that EGF/MCP-1 ratio significantly correlated with the eCrCl slope with a $P=0.017$.

DISCUSSION

Urinary cytokines seem to play a key role in the progression of renal damage in IgAN. EGF is the main trophic factor for tubular cells, and it has also been shown to modulate tissue response to injury.[13] Kidneys with tubulointerstitial damage exhibit a decreased EGF renal synthesis and urinary excretion.[14] Conversely, MCP-1 plays a major role in the progression of renal disease, both in animal models and in different types of human renal diseases, including IgAN.[15,16] In lupus nephritis, urinary levels of MCP-1 excretion significantly predicted both renal flares and the lack of response to therapy.[17] A recent study conducted in 215 patients with different nephropathies showed a correlation between urinary levels of MCP-1, interstitial monocyte infiltration, and albumin/creatinine ratio. In this study, urinary MCP-1 independently predicted renal survival.[18] In previous studies, we demonstrated in IgAN patients a correlation between urinary levels of MCP-1, monocytic infiltration, and tubulointerstitial damage,[12] and between urinary levels of EGF and the degree of histological lesions.[13]

This study is the first prospective clinical study evaluating the prognostic significance of urinary EGF/MCP-1 ratio on renal survival conducted in a cohort of incident IgAN patients with a long-term follow-up and comparing it with established risk factors for ESRD.

In the first part of the study, we demonstrated, as expected, a significant reduction of the EGF/MCP-1 ratio in the urine of patients with more severe histologic lesions at the time of renal biopsy. Then, we demonstrated a significant correlation between our biomarker, eCrCl, and uPr. Finally, we assessed the independent effect of EGF/MCP-1 ratio on adverse outcome. Not surprisingly, in our cohort of patients, we found that the severity of the histologic lesions, the amount of daily proteinuria, the presence of impaired renal function, and hypertension at the time of renal biopsy were all prognostic factors of the combined outcome at the univariate analysis, as demonstrated also in previous cohort studies.[3–6] The main finding in the multivariate analysis was

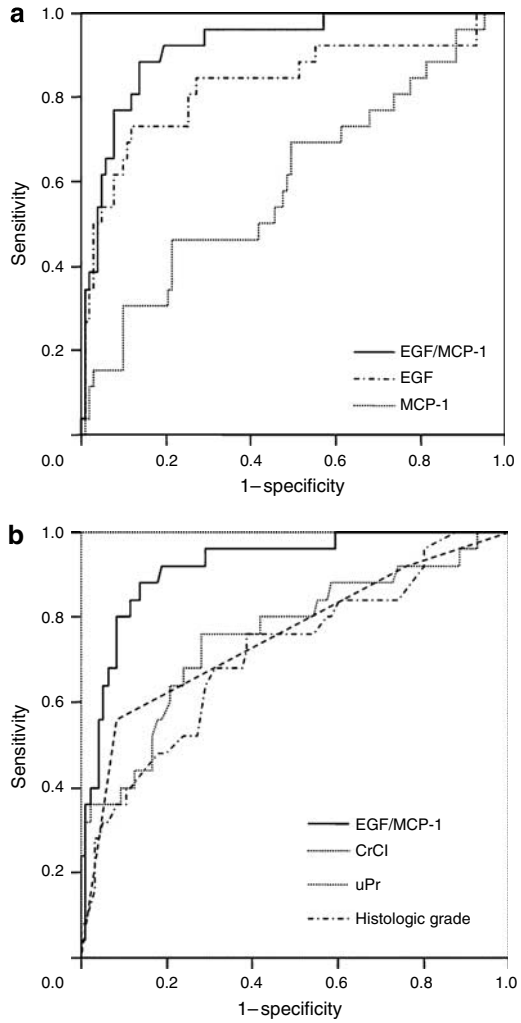

Figure 3 | ROC analysis. (**a**) ROC analysis of the predictive value for adverse outcomes of EGF, MCP-1, and EGF/MCP-1 ratio. The AUC of EGF/MCP-1 was 0.91 (95% CI: 0.84–0.95), showing a better capacity of discrimination compared to EGF (0.83, 95% CI: 0.76–0.89; $P = 0.01$) and MCP-1 (0.57, 95% CI: 0.49–0.66; $P < 0.001$) alone. (**b**) ROC analysis of the predictive value for adverse outcomes of EGF/MCP-1 ratio and other risk factors. The AUC of EGF/MCP-1 ratio was significantly higher than that of eCrCl (0.76 (0.65–0.88); $P = 0.002$), of histologic grade (0.75 (0.63–0.88); $P = 0.005$), and of uPr (0.68 (0.56–0.81); $P = 0.004$).

that the EGF/MCP-1 ratio, measured at the time of renal biopsy, independently predicted the combined outcome.

Although we demonstrated that the EGF/MCP-1 ratio was an independent risk factor for adverse outcome in our study population, this did not necessarily imply that it was able to accurately distinguish between patients with good prognosis and those with poor prognosis. For this reason, we also calculated the predictive value of EGF/MCP-1 ratio, of EGF and MCP-1 single measurements, and of the other prognostic factors by means of an ROC analysis. EGF/MCP-1 ratio improved the sensitivity and the specificity in predicting the adverse outcome compared to the two cytokines alone and to the other risk factors, as shown by the AUC in the ROC analysis (Figure 3a and b).

The EGF/MCP-1 ratio also significantly correlated with the rate of loss of eGFR in the univariate and multivariate analyses. These findings, confirmed in different multivariate approaches, show a strong association of the EGF/MCP-1 ratio and different outcome measures.

Selection bias was avoided in our study, by including all consecutive patients receiving a histologic diagnosis of IgAN in our renal unit and in a predefined period of enrollment (5 years). The demographic and clinical characteristics of the study population were comparable to those of our historic IgAN database, which includes more than 400 patients.[19]

However, this study has some limitations. The single-center nature of the report may have an impact on its external validity. In addition, renal survival in IgAN could be influenced by different therapeutic strategies. Considering that most of our patients received angiotensin converting enzyme inhibitors and/or angiotensin II receptor blockers during the follow-up, there were no statistically significant differences in the proportion of patients treated and those untreated in the three tertile groups of the EGF/MCP-1 ratio. For this reason, we do not think that therapy could have influenced our results. Another limitation could be the single measurement of cytokines at baseline and not during the follow-up, but this was the first time when we used the Renal Progression Kit, and after having obtained these results, we are planning to validate the method in a larger cohort of patients, with repeated measures of urine cytokines.

In conclusion, this study suggests that urinary EGF/MCP-1 ratio may be a useful and reliable marker of progression in IgAN. During the long-term follow-up of IgAN patients, the major difficulties are represented by the evaluation of disease progression with the routine laboratory parameters, which have great limitations in terms of sensitivity and specificity in monitoring the intrarenal changes. In these patients, it is sometimes necessary to repeat the renal biopsy, but this being an invasive procedure, a cost–benefit analysis is mandatory. In these particular cases, the EGF/MCP-1 ratio measurement might help us to predict the progressive deterioration of renal function, avoiding more invasive procedures when not necessary. These results could be used to prospectively identify high-risk patients who may benefit from a closer follow-up and a more aggressive therapeutic approach. Further prospective studies in a larger population of patients are warranted to support our hypothesis.

MATERIALS AND METHODS

Patients

This is a prospective cohort study. We enrolled all consecutive patients who underwent renal biopsy in our Renal Unit and received a diagnosis of IgAN from January 1995 to December 2000. The planned follow-up was to December 2005.

The included patients met the following criteria: (1) dominant or codominant deposition of mesangial IgA by immunofluorescence microscopy; (2) no signs or symptoms of secondary IgA deposition, including systemic lupus erythematosus, Schönlein–Henoch purpura, and liver disease. We excluded all those patients receiving corticosteroids or immunosuppressive therapy. All renal biopsies

were performed at least 30 days after episodes of macroscopic hematuria, after signed informed consent.

Serum creatinine and daily proteinuria (uPr) were assessed at baseline and over time. eCrCl was calculated with Cockcroft and Gault formula.[20] Patients were considered hypertensive if their arterial blood pressure was higher than the level recommended by the VI Joint National Committee Guidelines[21] or if levels less than 140–90 mm Hg were reached using antihypertensive drugs. Each patient was evaluated clinically and with laboratory tests every 3–6 months. Outcome measures were doubling of baseline sCr and/or ESRD (defined as the need for regular dialysis treatment or renal transplant). The study was performed according to recommendations outlined in the Declaration of Helsinki Principles (IV Adaptation).

Renal biopsies

Renal specimens, obtained by needle-core biopsies (14 or 16 gauge) performed under ultrasonographic guidance, were fixed in 4% formaldehyde, paraffin-embedded, and then processed for routine light microscopy stainings (hematoxylin–eosin, periodic acid-Schiff, silver methenamine, and Masson's trichrome). The frozen tissue was processed for routine immunofluorescence microscopy with antisera for IgG, IgA, IgM, C3, C1q, and fibrinogen (The Binding Site, Birmingham, UK).

On the basis of the severity of glomerular, tubular, and interstitial lesions, all renal biopsies were scored in five grades according to Lee's classification and grouped into mild lesions (grades I–II), moderate lesions (grades III), and severe lesions (grades IV–V).[22]

Urinary cytokine measurement

At the time of renal biopsy, morning urine samples were collected from each patient to evaluate urinary EGF and MCP-1 excretion. The urine samples were centrifuged at 600 *g* for 5 min and frozen at -20°C until tested. None of the patients had urinary tract infections at the time of the study. Quantitative assay of EGF and MCP-1 levels in urine samples was performed using the Renal Progression Kit (Apulia Biotech s.c.r.l., Valenzano (Bari), Italy). This is a direct sandwich enzyme-linked immunosorbent assay (ELISA) kit specific for the urine samples in which one-half of the polystyrene 96-well microtiter plate was coated with monoclonal anti-human MCP-1 antibody (Anogen, Mississauga, Ontario, Canada) and the other half with monoclonal anti-human EGF antibody (Anogen). After incubation with urine, the second monoclonal antibody (Anogen) conjugated with horseradish peroxidase (HRP) was added to the respective wells and the enzymatic reaction was detected in an automatic microplate photometer (Programmable MTP reader DV 990BV6; Gio. DeVita E C, Rome, Italy). The EGF and MCP-1 concentrations of the unknown urine samples were determined by interpolation into a standard curve developed with known amounts of recombinant human EGF and MCP-1 proteins (R&D Systems Inc., Minneapolis, MN, USA), and expressed in ng ml^{-1}. The lower detection limit was 0.03 ng ml^{-1} for EGF and 0.42 ng ml^{-1} for MCP-1. The measurement of each cytokine was normalized for uCr, and then we used the ratio of the two normalized measurements as a marker of renal damage. The intra- and interassay coefficients of variation were approximately 7 and 8% for EGF and 5 and 8% for MCP-1, respectively.

Epidermal growth factor and MCP-1 stability in the urine was tested by measuring the concentrations of the two cytokines at the time of collection and after 2 months, and we did not observe any significant difference between the two measurements. Moreover, we measured urine EGF/MCP-1 ratio in four normal subjects over a period of 4 consecutive days, demonstrating a daily variability of 7.8%. The evaluation of EGF and MCP-1 urine excretion was performed by an independent researcher unaware of patient's clinical history.

Statistical analysis

Continuous data were analyzed descriptively by using mean $\pm$ s.d. or median and interquartile range. Categorical data were described as absolute frequencies and percentages.

Baseline characteristics of individuals affected by IgAN were compared by one-way analysis of variance, Kruskal–Wallis test, unpaired Student's *t*-test, and Mann–Whitney *U*-test as appropriate for continuous data, and by the χ^2 statistic for categorical data. To evaluate the differences between patients, the values of urinary EGF/MCP-1 ratio were grouped into tertiles.

Linear regression analysis was performed to determine the correlation between different levels of urinary EGF, MCP-1, and the EGF/MCP-1 ratio and the renal function expressed as eCrCl. We also investigated the correlation between these cytokines and the degree of uPr at the time of renal biopsy. Finally, the correlation between the EGF/MCP-1 ratio and the loss of GFR expressed as eCrCl slope was explored by simple linear and a multivariate regression analysis, the last adjusting for other risk factors.

Cumulative renal survival from the time of renal biopsy was analyzed by Kaplan–Meier curves for censored data, using as outcome measure the combined end point of doubling sCr and/or ESRD; differences between groups were compared by the log-rank test.

The association between the combined outcome and potential risk factors was examined using univariate Cox's regression proportional hazard method. Predictors univariately associated with outcome ($P<0.10$) were included in a multivariate Cox's regression model, and the goodness of fit was estimated. We used all predictors as continuous variables. Risk estimates were presented as unadjusted and adjusted hazard ratios, and their 95% CIs were calculated by an estimated regression coefficient in the Cox's regression analysis.

Since all covariates used in the final model were strongly correlated with the exposure variable, to address the possibility that they could be potential confounders, we tested for the significance of interactions between the EGF/MCP-1 ratio and the covariates included in the multivariable model.

The problem of missing values was resolved by carrying out a complete case analysis; data were missing in less than 5% of patients.

The predictive value of the known risk factors EGF, MCP-1, and the EGF/MCP-1 ratio, used as test variables in predicting the combined end point was investigated by the area under the ROC, using each parameter as a continuous variable.

All analyses were performed using SPSS for Windows, release 12.0; results were considered statistically significant when two-tailed *P*-values were $\leqslant 0.05$.

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REFERENCES

1. Berger J, Hinglais N. Les depots intercapillaires d'IgA-IgG. *J Urol Nephrol (Paris)* 1968; **74**: 694–695.
2. Schena FP, Coppo R. IgA nephropathies. In: Davison AM, Cameron JS, Grunfeld JP, Ponticelli C, Ritz E, Winearls CG, van Ypersele C (eds). *Oxford Textbook of Clinical Nephrology*. Oxford University Press: New York, 3rd edn 2004, pp 470–501.
3. D'Amico G, Minetti L, Ponticelli G *et al.* Prognostic indicators in idiopathic IgA mesangial nephropathy. *Q J Med* 1986; **59**: 363–378.
4. Alamartine E, Sabatier JC, Guerin C *et al.* Prognostic factors in mesangial IgA glomerulonephritis: an extensive study with univariate and multivariate analyses. *Am J Kidney Dis* 1991; **18**: 12–19.
5. Katafuchi R, Oh Y, Hori K *et al.* An important role of glomerular segmental lesions on progression of IgA nephropathy: a multivariate analysis. *Clin Nephrol* 1994; **41**: 191–198.
6. Bartosik LP, Lajoie G, Sugar L, Cattran DC. Predicting IgA nephropathy. *Am J Kidney Dis* 2001; **38**: 728–735.
7. Schainuck LI, Striker GE, Cutler RE, Benditt EP. Structural-functional correlations in renal disease. II. The correlations. *Hum Pathol* 1970; **1**: 631–641.
8. Segerer S, Nelson PJ, Schlondorff D. Chemokines, chemokine receptors, and renal disease: from basic science to pathophysiologic and therapeutic studies. *J Am Soc Nephrol* 2000; **11**: 152–176.
9. Eddy AA. Molecular basis of renal fibrosis. *Pediatr Nephrol* 2000; **15**: 290–301.
10. Remuzzi G, Bertani T. Pathophysiology of progressive nephropathies. *N Engl J Med* 1998; **339**: 1448–1456.
11. Eddy AA. Role of cellular infiltrates in response to proteinuria. *Am J Kidney Dis* 2001; **37**(Suppl 2): S25–S29.
12. Grandaliano G, Gesualdo L, Ranieri E *et al.* Monocyte chemotactic peptide-1 expression in acute and chronic human nephritides: a pathogenetic role in interstitial monocytes recruitment. *J Am Soc Nephrol* 1996; **7**: 906–913.
13. Gesualdo L, Di Paolo S, Calabro A *et al.* Expression of epidermal growth factor and its receptor in normal and diseased human kidney: an immunohistochemical and *in situ* hybridization study. *Kidney Int* 1996; **49**: 656–665.
14. Ranieri E, Gesualdo L, Petrarulo F, Schena FP. Urinary IL-6/EGF ratio: a useful prognostic marker for the progression of renal damage in IgA nephropathy. *Kidney Int* 1996; **50**: 1990–2001.
15. Tomino Y, Tsuge T, Suzuki Y *et al.* Basic research in progressive glomerulopathies: the role of fibrosing factors in IgA nephropathy and diabetic nephropathy. *Kidney Int Suppl* 2005; **94**: S92–S95.
16. Yokoyama H, Wada T, Furuichi K *et al.* Urinary levels of chemokines (MCAF/MCP-1, IL-8) reflect distinct disease activities and phases of human IgA nephropathy. *J Leukoc Biol* 1998; **63**: 493–499.
17. Rovin BH, Song H, Birmingham DJ *et al.* Urine chemokines as biomarkers of human systemic lupus erythematosus activity. *J Am Soc Nephrol* 2005; **16**: 467–473.
18. Eardley KS, Zehnder D, Quinkler M *et al.* The relationship between albuminuria MCP-1/CCL2, and interstitial macrophages in chronic kidney disease. *Kidney Int* 2006; **69**: 1189–1197.
19. Manno C, Strippoli GFM, D'Altri C *et al.* A novel, simpler histological classification for renal survival in IgA nephropathy: a retrospective study. *Am J Kidney Dis* 2007; **49**: 763–775.
20. Cockcroft DW, Gault MH. Prediction of creatinine clearance from serum creatinine. *Nephron* 1976; **16**: 31–41.
21. The sixth report of the Joint National Committee on Prevention, Detection, Evaluation, and Treatment of high blood pressure. *Arch Int Med* 1997; **157**: 2413–2446.
22. Lee S-MK, Rao VM, Franklin WA *et al.* IgA nephropathy: morphologic predictors of progressive renal disease. *Hum Pathol* 1982; **13**: 314–322.