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PROGNOSTIC SIGNIFICANCE OF GLOMERULAR AND TUBULOINTERSTITIAL MORPHOMETRY IN IDIOPATHIC MEMBRANOUS NEPHROPATHY

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SUMMARY

The purpose of our study was to investigate the prognostic value of clinical and pathological, in particular glomerular and tubulointerstitial morphometric variables in idiopathic membranous nephropathy. We prospectively followed 60 Caucasian patients diagnosed with idiopathic membranous nephropathy for at least 2 years or until primary outcome (>=50% permanent decrease in estimated glomerular filtration rate or death). Glomerular and tubulointerstitial morphometric variables at the time of renal biopsy were analyzed with respect to this outcome. Univariate analysis revealed that significant negative prognostic factors for this outcome were higher cholesterol and smaller albumin concentrations, higher creatinine and maximal 24-hour proteinuria, higher grade of nephroangiosclerosis, higher glomerular basement membrane thickness and glomerulopathy index, higher interstitial fibrosis and tubular atrophy percentage and higher injury score. In multivariate analysis, only the maximal 24-hour proteinuria and interstitial fibrosis and tubular atrophy percentage were independent predictors of this outcome. The results suggest that morphometric analysis, mainly quantitative measurement of interstitial fibrosis and tubular atrophy percentage, injury score, glomerular basement membrane thickness and glomerulopathy index could be used as an additional method for risk stratification of patients with idiopathic membranous nephropathy.

INTRODUCTION

Idiopathic membranous nephropathy (IMN) is one of the most common primary glomerulonephritides, accounting for 9.7% to 29.4% [3, 9, 17]. It is considered the most common cause of nephrotic syndrome in adults [22, 25]. It has a very variable clinical course with all possible outcomes, ranging from spontaneous remission, with a reported incidence between 32 and 67%, to progressive deterioration and development of end-stage renal disease (ESRD), with a reported incidence between 12 and 44% [7, 18, 21, 26]. Considering a variable clinical course, identification of specific and sensitive prognostic factors is of great importance for the selection of patients undergoing immunosuppressive treatment. Numerous prognostic factors have been validated in IMN, and for most of them, low specificity and/or sensitivity was found [4, 7, 12, 16, 20, 22, 25, 27]. Glomerular and tubulointerstitial morphometric analysis is being used as a complementary method of routine analysis of renal biopsy in various diseases [23]. Regarding IMN, only a few studies used morphometric analysis [1, 19, 24]. The aim of this study was to validate glomerular and tubulointerstitial morphometric prognostic factors, as well as clinical factors, in our cohort of patients with IMN.

MATERIAL AND METHODS

We included patients having undergone kidney biopsy in two Nephrology Departments in Zagreb, Croatia, between 1996 and 2009, and diagnosed with IMN. Patients with secondary forms of membranous nephropathy were excluded from the study. Age, gender, arterial blood pressure, serum creatinine, estimated glomerular filtration rate (EGFR), calculated according to the CKD-EPI formula [14], serum cholesterol, albumin and maximal 24-hour proteinuria (until the biopsy) were recorded at the time of biopsy. In all patients, kidney biopsy was performed, and all specimens were processed for light, immunofluorescence and electron microscopy using

standardized techniques. The histopathological parameters analyzed were as follows: Ehrenreich and Churg disease stage I to IV [6], semiquantitatively defined nephroangiosclerosis grade (0-none, 1-mild, 2-moderate, 3-severe), immunoglobulin G deposition grade (0-lack of deposition, 1-mild, 2-moderate, 3-severe), complement C3 deposition grade (0-lack of deposition, 1-mild, 2-moderate, 3-severe), presence of secondary focal segmental glomerulosclerosis (FSGS) and heterogenicity of immune deposits (synchronous electron dense deposits with a single stage in all analyzed glomeruli were arbitrarily classified as homogenous type and others having various stages as heterogeneous type, according to Yoshimoto et al.) [30]. Morphometric analysis was carried out by a semiautomatic image analysis procedure, using the optical microscope Olympus BX41 with camera Olympus DP71 connected with PC and with ImageJ image analysis software (http://rsb.info.nih.gov/ij/). Glomerular morphometry was carried out by analysis of light microscopy (PAS-stained images with a magnification of x400). In each case, 5-10 glomeruli, cut through hilum or having complete outline of Bowman's capsule, were selected for glomerular morphometry. Biopsies with less than 5 glomeruli were not included in the study. After opening the image, ImageJ measurement tools were calibrated by micrometer specific to the magnification into standard units (mm, μm and nm). Glomerular morphometric parameters measured were: glomerular diameter (GD), tuft diameter (TD), glomerular area (GA), tuft area (TA), mesangial matrix and membranes area (MA), urinary space area (UA=GA-TA), capillary space area (CA=TA-MA), tuft volume fraction (TVF=(TA/GA)x100), urinary space volume fraction (UVF=(UA/GA)x100), membranes and mesangial matrix volume fraction (MVF=(MA/GA)x100) and capillary space volume fraction (CVF=(CA/GA)x100), as described earlier by Rayath et al. [24]. After obtaining color image by Image/Color/Split channels tool, red, green and blue channels of the image were separated, and for further analysis, the green channel was kept, because it gives the sharpest glomerular image. Using a free-hand tool from the menu

bar and tracing the outline of the glomerulus and then the tuft, an area was selected as region of interest (ROI), and then, using Analyze/Measure tool, GA, TA and MA measured. MA was measured by Image/Type/8-bit tool to convert the green channel of the original image to grayscale, and then the threshold for staining detection was set by selecting Image/Adjust/Threshold tool. The final grayscale image was created in which black areas approximately represent mesangial matrix and membrane areas (MA), as reported earlier by Rayath et al. [24]. For every glomerular morphometric parameter measured, the mean of all values measured in a single biopsy was used as reference value for the individual patient. Morphometric measurement of interstitial fibrosis and tubular atrophy (IFTA) was carried out by analyzing Masson-trichrome stained images with a magnification of x400 (areas of fibrosis are stained blue). After separating the glomerules and medulla from the cortex, blue color was defined as ROI by selecting one blue area with a freehand tool and then by clicking the Image/Adjust/Color threshold tool and Sample button, which removed pixels not falling into the selected color range. After that, the image is converted into binary (8-bit), and the whole biopsy cylinder is marked as ROI. Then, using Analyze Particles tool, the Area fraction was determined which represents IFTA (in percentage) (described earlier in detail by Rangan and Tesch) [23]. Injury score (IS) is a marker of chronic damage and has recently been shown to have prognostic value in focal segmental glomerulosclerosis [29]. It is calculated as IS = (number of segmental sclerotized glomeruli + number of globally sclerotized glomeruli)/total number of glomeruli + IFTA (expressed as an absolute number). Electron microscopy was carried out using JEOL JEM-1400 electron microscope. Glomerular basement membrane thickness (GBMT) was ascertained on images at a magnification x8000. GBMT was determined as a harmonic mean of 100 orthogonal intercepts across the glomerular basement membrane (GBM) measured from at least 5 glomerular capillary loops by line tool of ImageJ software on the acquired images after

calibration for magnification. Harmonic mean was multiplied by $8/3\pi$ to correct the measuring error due to oblique sectioning of capillary walls [13, 24]. In each measurement, GBMT was defined as a distance between endothelial cell and podocyte membrane, and included intramembranous immune deposits. Glomerulopathy index (GPI) was calculated by the formula GPI=1/10xGBMT+MVF (according to Rayat et al.) [24].

Follow-up started at the time of biopsy. It was minimally 2 years and continued until February 2011 or until the primary outcome. Serum creatinine and 24-hour proteinuria were measured every 3 months during follow-up, and EGFR was calculated. Combined primary outcome was renal failure (RF, defined as >=50% permanent decrease in EGFR from baseline values) or death.

Statistical analysis was performed using the SPSS version 17.0 for Windows and MedCalc version 12.2. Normally distributed variables were expressed as mean±standard deviation and compared using Student's t-test. Nonparametric continuous variables were expressed as median and interquartile range and compared using Mann-Whitney U test. Categorical variables were expressed in percentage and compared using χ^2-test or Fischer's exact test. Univariate comparisons for outcomes were performed by Kaplan-Meier curves and log-rank test. Receiver operating characteristics (ROC) curves analysis was made to determine area under curve (AUC) and to calculate the sensitivity and specificity of various clinical and morphometric baseline variables in the prediction of primary outcome, using the most discriminative thresholds (cut-off values). A multivariate Cox proportional hazard model was constructed to determine independent variables associated with primary outcome. Only variables associated by univariate analysis were included in a multivariate model. For all analyses, $p<0.05$ was considered significant.

RESULTS

Sixty Caucasian patients were included in this study. Nephrotic syndrome was present in 93.3% of the patients. Tables 1, 2 and 3 show the baseline clinical, histological and morphometric parameters with respect to primary outcome. The patients were treated nonrandomly, following guidelines [2, 5, 8, 22]; 85% of the patients with immunosuppressives (56.7% with glucocorticoid + alkylating agent, 18.3% with glucocorticoid+cyclosporin and 10% with glucocorticoid alone), and 90% of the patients received renin-angiotensin inhibiting drugs. Patients were followed for a median of 48 months (range 6 to 132 months). During follow-up, 12 patients reached primary outcome (20%), two patients died (one of thromboembolic incident; for the other one, the cause of death was unknown). The estimated probability of survival without primary outcome was 79.0±6.8% at 60 months and 62.7±10.0% at 84 months (Kaplan-Meier survival analysis). In univariate analysis, higher serum creatinine (lower EGFR), higher serum cholesterol and lower albumin concentration were associated with primary outcome. Pathohistological and morphometric variables associated with primary outcome were higher nephroangiosclerosis grade, higher GBMT and GPI, higher IFTA percentage and higher IS. Other morphometric and pathohistological variables tested were not associated with primary outcome. The ROC analysis showed that the most discriminative variables in the prediction of primary outcome were IFTA and IS (Table 4). The optimal cut-off value of IFTA was 18%, and that of IS 0.322 (Figs. 1 and 2). Kaplan-Meier survival analysis showed that renal and patient survival (primary outcome) was significantly higher in patients with IFTA<=18% (Fig. 3) and IS<=0.322 (Fig. 4). Cox proportional hazards model included variables selected by univariate analysis. The results are shown in Table 5. The only independent predictors of primary outcome were maximal 24-hour proteinuria (hazard ratio, HR=1.127) and IFTA (HR=1.029). We also created a similar Cox proportional hazards model with IFTA as a categorical variable, using cut-

off values on the basis of ROC analysis and AUC. The newe model remained statistically stable also, showing that patients with IFTA>18% had HR=17.662 for primary outcome (95% confidence interval 2.235-139.581; p=0.006) compared to patients with IFTA<=18%. Because our study may be biased due to nonrandomized immunosuppressive therapy, we additionally performed the ROC analysis, including only patients given immunosuppressive therapy, and the results for IFTA and IS were similar (cut-off for IFTA>18%, AUC=0.854, sensitivity 90.0%, specificity 75.61%, p<0.0001).

DISCUSSION

There are numerous studies with a focus on prognostic factors in IMN, but their results vary considerably. The reason for this is most probably due to a great variation in inclusion criteria and in the outcomes evaluated, as stipulated by Marx et al. [15]. Consequently, there is a large diversity in the results of the prognostic factors studied, so that for virtually every prognostic factor investigated, there are studies demonstrating or refuting its significance in IMN [15, 25]. This study attempted to overcome some shortcomings of previous studies by focusing on glomerular and tubulointerstitial morphometric variables in a well-defined cohort of patients with IMN. Our outcome was defined as proposed by Marx et al. [15].

It is interesting that in our study, gender and age were not found to be statistically significant predictors of RF. This corroborates the findings of most recent studies in which age and male gender were not significant predictors of outcome as well [28, 30]. In the majority of studies, as well as in ours, hypertension was not found to be an independent predictor of RF [22, 25]. Only Heeringa et al. [10] reported that diastolic blood pressure is an unfavorable predictor for RF (but defined as serum creatinine >135μmol/l). The prognostic significance of renal function (measured by serum creatinine and/or EGFR) at the time of diagnosis is potentially

biased because of a transitory decrease in GFR in patients with severe nephrotic syndrome, and because of the fact that a shorter renal survival time in patients with permanently decreased baseline renal function may result from baseline chronic kidney injury that may not be a consequence of the IMN itself, as shown by Troyanov et al. [28]. In line with that, we found renal function to be associated with primary outcome only in univariate, but not in multivariate analysis. Similar results were published by Marx et al. [16] and Yoshimoto et al. [30], while Heeringa et al. [10] reported serum creatinine as an independent predictor. We found baseline 24-hour proteinuria as an unfavorable predictor for RF in univariate analysis and multivariate analysis, which is consistent with other studies [11, 16, 30].

The main focus of our study was the evaluation of morphometric variables in the prognosis of IMN. We found IFTA, measured quantitatively, as a significant predictor for renal and patient survival in both univariate and multivariate analysis. In the present study, some other histological indices (GBMT, GPI and IS) were significantly associated with progression of renal dysfunction only in univariate analysis, while morphometric variables related to glomerular size and volume fractions, as well as immunoglobulin and complement deposition grade and FSGS, were not found to be predictive of RF. A predictive value of quantitatively measured IFTA demonstrating that even low grade of IFTA (IFTA>18%) was associated with a significantly worse renal outcome, independent of immunosuppressive therapy, is the major finding of our study. We believe that this does not implicate that patients with IFTA>18% should not be treated with immunosuppressives, but it rather reflects possible additional non-immunological mechanisms of disease progression. It is important to recall that in the present study, virtually all patients were treated with angiotensin-blocking drugs. Thus, additional possible mechanisms of IMN progression, besides inflammation and the renin-angiotensin pathway, should be the target of future studies. Follow-up renal biopsies may be one of the tools for studying mechanisms of

the disease progression, as well as the effect of established drugs and potential new drugs. In most recent studies, IFTA was found to be an unfavorable prognostic factor for the outcomes of ESRD and RF only in univariate analysis [10, 16, 28, 30]. In multivariate analysis, IFTA was found to be a negative predictive factor only in the studies by Yoshimoto et al. [30] and Paraskevakou et al. [19]. It is important to note that in the latter study, IFTA was measured quantitatively as well. In the study conducted by Yoshimoto et al. [30], only a relatively small percentage of patients were treated by the standard immunosuppressive therapy, making a comparison with the present study difficult.

Our study is limited because of the relatively small number of patients and the fact that it was not a randomized study. In addition, the fact that this study is the second to evaluate the prognostic significance of quantitatively determined IFTA implicates the need for a re-evaluation of our results (especially a low cut-off IFTA value for progression of renal disease) in future prospective studies. If quantitative IFTA measurement proves to be a more reliable marker of disease progression than semi-quantitative measurement, it would be easy to implement it. The tools used for quantifying IFTA are readily available (like ImageJ that we used), but in clinical practice, an automated high throughput system using whole-slide imaging would allow even a faster analysis, as well as the translation of the results of our study in the future.

In conclusion, following the evaluation of several prospective prognostic factors of IMN progression, our study identified quantitatively measured IFTA as the most predictive one. If the present findings are confirmed, they could be easily translated into a routine method of biopsy analysis in IMN.

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TABLES

Table 1. Clinical data at baseline (values are given as mean±SD for normally distributed continuous variables and as median with interquartile range for nonparametric continuous variables)

		PRIMARY OUTCOME (RF† or death)		
	All patients (n=60)	NO (n=48)	YES (n=12)	p
Age (years)	**52.4±13.8**	**51.31±14.13**	**56.67±11.67**	**NS**
Gender (female/male, %)	**40/60**	**35.4/64.6**	**58.3/41.7**	**NS**
Hypertension (%)	**73.3**	**70.8**	**83.3**	**NS**
Systolic blood pressure (mmHg)	**151.07±29.04**	**148.52±28.18**	**161.25±31.42**	**NS**
Diastolic blood pressure (mmHg)	**91.48±12.87**	**90.71±12.93**	**94.58±12.70**	**NS**
MAP‡ (mmHg)	**111.12±16.53**	**109.67±16.18**	**116.95±17.35**	**NS**
Serum Creatinine (μmol/l)	**104.97±52.33**	**97.58±43.91**	**134.50±72.55**	**0.043***
EGFR§ (ml/min)	**75.28±28.54**	**79.94±26.76**	**56.65±28.93**	**0.010***
Serum Cholesterol (mmol/l)	**8.25 (6.15-12.07)**	**8.13 (6.00-11.15)**	**11.67 (7.80-13.05)**	**0.021****
Serum Albumin (g/l)	**25.15±8.30**	**26.44±8.12**	**19.98±7.17**	**0.015***
Maximal 24-hour proteinuria ($g/1.73m^2$)	**7.74 (5.61-12.62)**	**7.15 (4,71-12.19)**	**12.90 (7.68-17.35)**	**0.008****
Immunosupressive therapy (%) yes / no	**85.0 / 15.0**	**85.4 / 14.6**	**83.3 / 16.7**	**NS**

* - Student t – test; ** - Mann-Whitney U – test; †RF=renal failure (>=50% permanent decrease in EGFR from baseline); ‡MAP=mean arterial pressure; §EGFR=estimated glomerular filtration rate.

Table 2. Basic light, immunofluorescence and electron microscopy findings

		PRIMARY OUTCOME (RF† or death)		
	All patients (n=60)	NO (n=48)	YES (n=12)	p
Ehrenreich-Churg Stage (%) I / II / III / IV	**13.3/43.3/28.3/15.1**	**16.7/41.7/29.1/12.5**	**0.0/50.0/25.0/25.0**	**NS**
Ehrenreich-Churg Stage (%) I or II / III or IV	**56.6 / 43.4**	**58.3 / 41.7**	**50.0 / 50.0**	**NS**
Grade of IgG deposition (%) 0 / 1 / 2 / 3	**1.7/23.3/38.3/36.7**	**0.0/27.1/35.4/37.5**	**8.4/8.3/50.0/33.3**	**NS**
Grade of C3 deposition (%) 0 / 1 / 2 / 3	**11.7/51.7/25.0/11.7**	**12.5/54.2/25.0/8.3**	**8.3/41.7/25.0/25.0**	**NS**
Heterogenous immune deposits (%)	**51.7**	**47.9**	**66.7**	**NS**
Grade of nephroangiosclerosis (%) 0 / 1 / 2 / 3	**45.0/31.7/15.0/8.3**	**50.0/35.4/10.4/4.2**	**25.0/16.7/33.3/25.0**	**0.013***
Secondary FSGS§ (%)	**56.7**	**54.2**	**66.7**	**NS**

*-χ^2 – test; †RF=renal failure (>=50% permanent decrease in EGFR from baseline); ‡MAP=mean arterial pressure; §FSGS=focal segmental glomerulosclerosis.

Table 3. Morphometric data at baseline (Continuous variables values are given as mean±SD for normally distributed variables and as median with interquartile range for non-normally distributed variables)

	All patients (n=60)	PRIMARY OUTCOME (RF† or death)		
		NO (n=48)	YES (n=12)	p
Glomerular area (GA, mm^2)	**0.040±0.009**	**0.039±0.008**	**0.041±0.012**	**NS**
Tuft area (TA, mm^2)	**0.032±0.007**	**0.032±0.007**	**0.033±0.010**	**NS**
Mesangial area (MA, mm^2)	**0.015±0.004**	**0.015±0.003**	**0.016±0.005**	**NS**
Urinary space area (UA, mm^2)	**0.008±0.003**	**0.008±0.002**	**0.009±0.003**	**NS**
Capillary space area (CA, mm^2)	**0.017±0.004**	**0.017±0.004**	**0.017±0.005**	**NS**
Glomerular diameter (GD, mm)	**0.231±0.029**	**0.229±0.027**	**0.237±0.038**	**NS**
Tuft diameter (TD, mm)	**0.209±0.029**	**0.209±0.027**	**0.213±0.038**	**NS**
Tuft volume fraction (TVF, in %)	**80.093±4.518**	**80.440±4.264**	**78.706±5.400**	**NS**
Urinary space volume fraction (UVF, in %)	**19.907±4.518**	**19.560±4.264**	**21.294±5.400**	**NS**
Mesangial volume fraction (MVF, in %)	**37.576±4.270**	**37.524±4.472**	**37.783±3.506**	**NS**
Capillary space volume fraction (CVF, in %)	**42.517±5.310**	**42.916±5.301**	**40.923±5.264**	**NS**
Glomerular basement membrane thickness (GBMT, nm)	**882.258±335.133**	**847.320±337.270**	**1016.187±303.272**	**0.049***
Glomerulopathy index (GPI)	**125.858±34.424**	**122.325±35.344**	**139.402±27.891**	**0.027***
Interstitial fibrosis and tubular atrophy (IFTA, in %)	**10.0 (8.0-32.75)**	**10.00 (5.50-16.50)**	**38.40 (30.00-55.00)**	**<0.000****
Injury score (IS)	**0.274 (0.165-0.514)**	**0.239 (0.141-0.374)**	**0.555 (0.339-0.922)**	**0.001****

* - Student t – test; ** - Mann-Whitney U – test; †RF=renal failure (>=50% permanent decrease in EGFR from baseline).

Table 4. Sensitivity, specificity, PPV[†] and NPV[‡] of the most discriminate threshold levels of significant clinical and morphometric parameters in the prediction of primary outcome (RF[§] or death)

Parameter	AUC[¶]	Threshold (cut-off)	Sensitivity (%)	Specificity (%)	PPV	NPV	p
Interstitial fibrosis and tubular atrophy (%)	**0.872**	**>18.0**	**91.67**	**77.08**	**50.0**	**97.4**	**<0.001**
Injury score (IS)	**0.829**	**>0.322**	**91.67**	**75.0**	**47.8**	**97.3**	**<0.001**
Glomerulopathy index (GPI)	**0.688**	**>124.154**	**75.0**	**63.04**	**34.6**	**90.6**	**0.046**
Maximal 24-hour proteinuria (g)	**0.747**	**>7.2**	**83.33**	**54.17**	**31.3**	**92.9**	**0.012**
Estimated glomerular filtration rate (ml/min)	**0.729**	**≤51.51**	**58.33**	**85.42**	**50.0**	**89.1**	**0.015**
Serum albumine (g/l)	**0.719**	**≤27.2**	**91.67**	**43.75**	**28.9**	**95.5**	**0.020**
Serum cholesterol (mmol/l)	**0.716**	**>11.0**	**66.7**	**70.0**	**40.0**	**90.0**	**0.021**
Serum creatinine (µmol/l)	**0.690**	**>102.0**	**58.33**	**81.25**	**43.8**	**88.6**	**0.043**

[†]PPV= positive predictive value; [‡]NPV=negative predictive value; [§]RF=renal failure (>=50% permanent decrease in estimated glomerular filtration rate from baseline); [¶]AUC=area under curve.

Table 5. The multivariate Cox proportional hazards model for the association of potentially predictive variables with primary outcome (RF[†] or death)

	Hazard ratio	95% CI[‡]	p
Serum Cholesterol (mmol/l)	**1.015**	**0.719-1.433**	**0.932**
Serum Albumin (g/l)	**0.886**	**0.771-1.019**	**0.090**
Estimated glomerular filtration rate (ml/minute)	**0.974**	**0.943-1.005**	**0.103**
Maximal 24-hour proteinuria (g)	**1.127**	**1.011-1.053**	**0.008**
Interstitial fibrosis and tubular atrophy (%)	**1.029**	**1.007-1.051**	**0.010**
Injury score	**0.123**	**0.002-6.944**	**0.308**
Glomerular basement membrane thickness (nm)	**0.992**	**0.973-1.012**	**0.453**
Glomerulopathy index	**1.062**	**0.876-1.288**	**0.537**
Nephroangiosclerosis grade (2 or 3 vs. 0 or 1)	**1.972**	**0.337-11.554**	**0.451**

[†]RF = >=50% permanent decrease in estimated glomerular filtration rate during follow-up; [‡]CI=confidence interval.

FIGURES

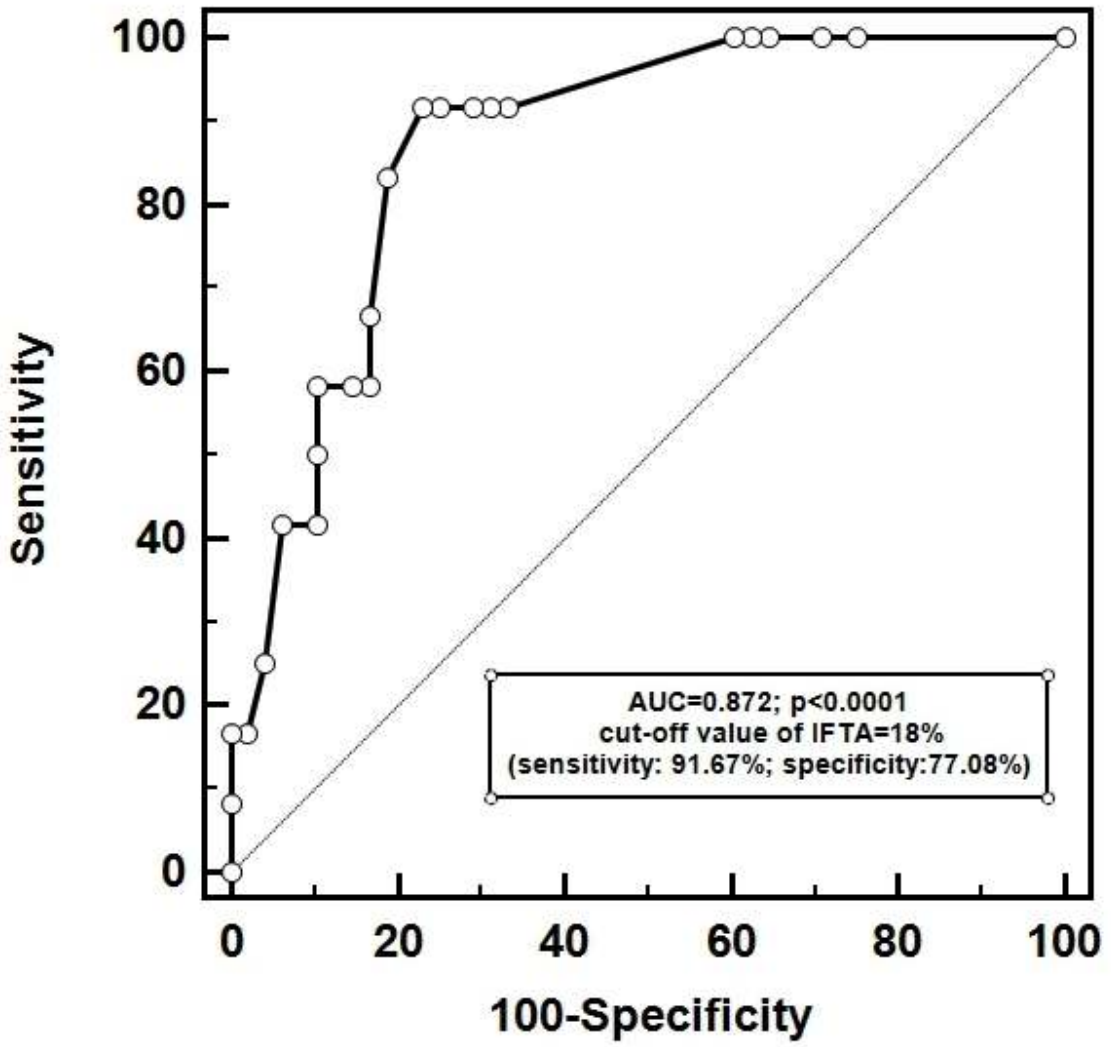

Figure 1. Receiver operating characteristics (ROC) curves analysis for evaluating cut-off value of optimal interstitial fibrosis and tubular atrophy (IFTA) percentage to predict primary outcome (>=50% permanent decrease in estimated glomerular filtration rate during follow-up or death).

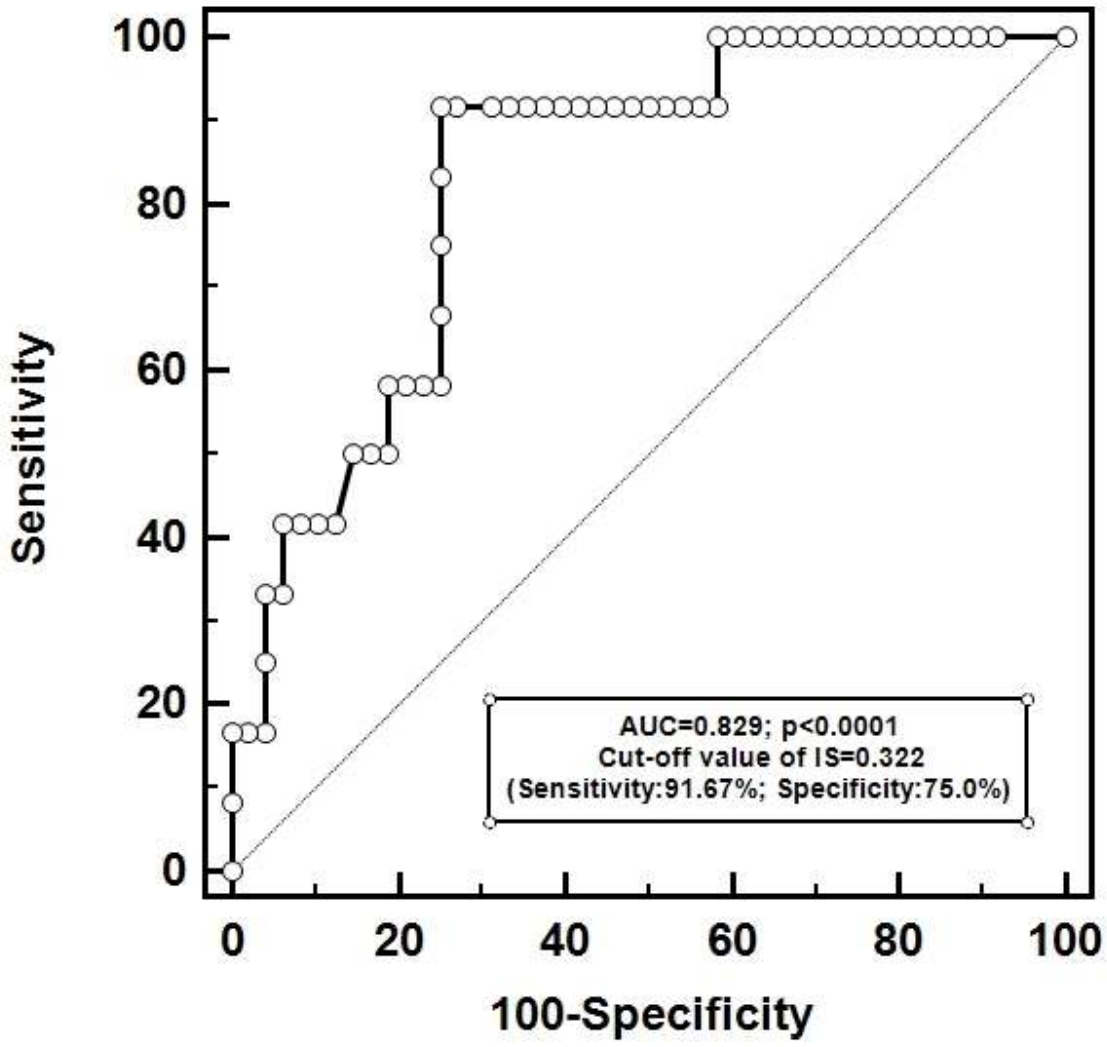

Figure 2. Receiver operating characteristics (ROC) curves analysis for evaluating cut-off value of optimal injury score (IS) to predict primary outcome (>=50% permanent decrease in estimated glomerular filtration rate during follow-up or death).

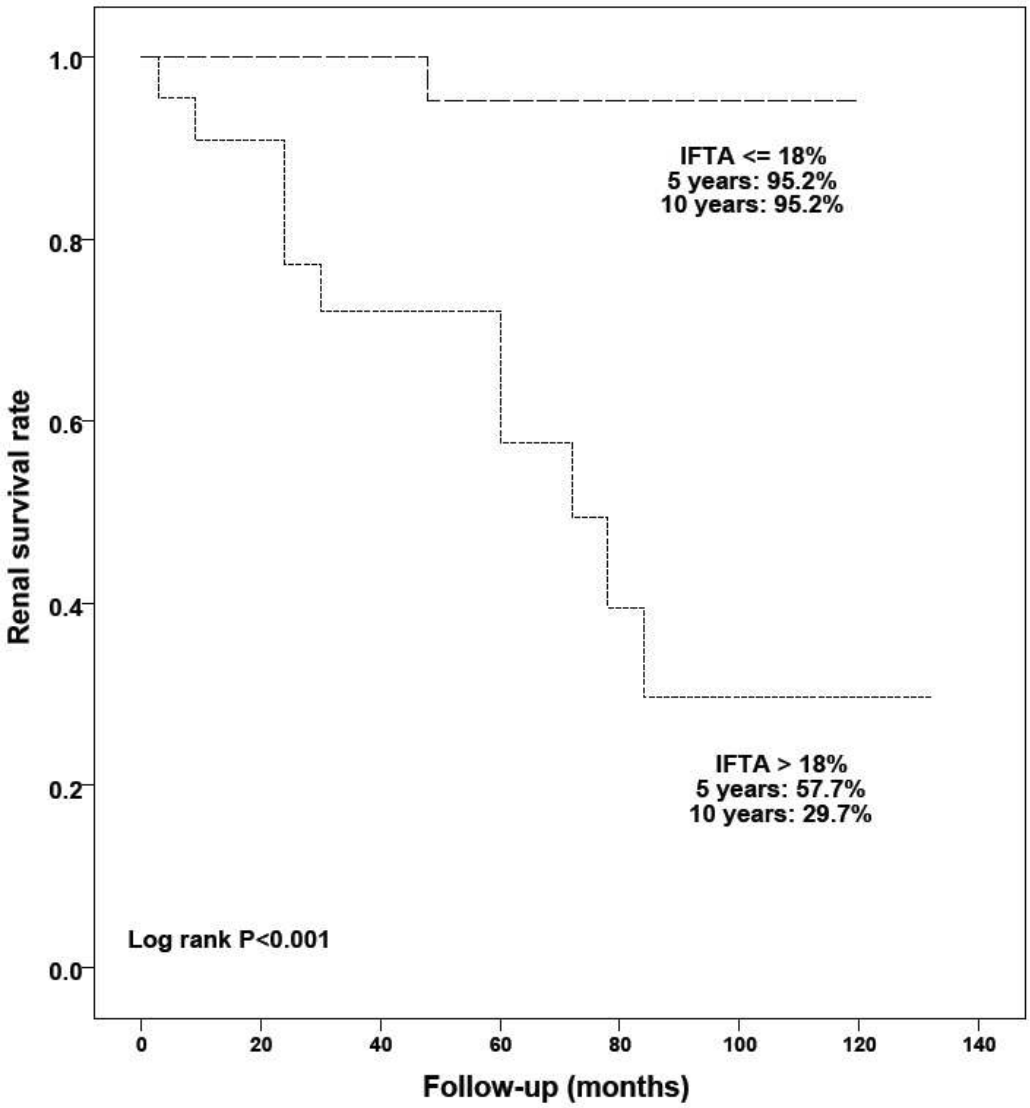

Figure 3. Renal and patient survival rate by Kaplan-Meier survival analysis and log-rank test of patients in the groups with interstitial fibrosis and tubular atrophy (IFTA) percentage >18% or <=18%.

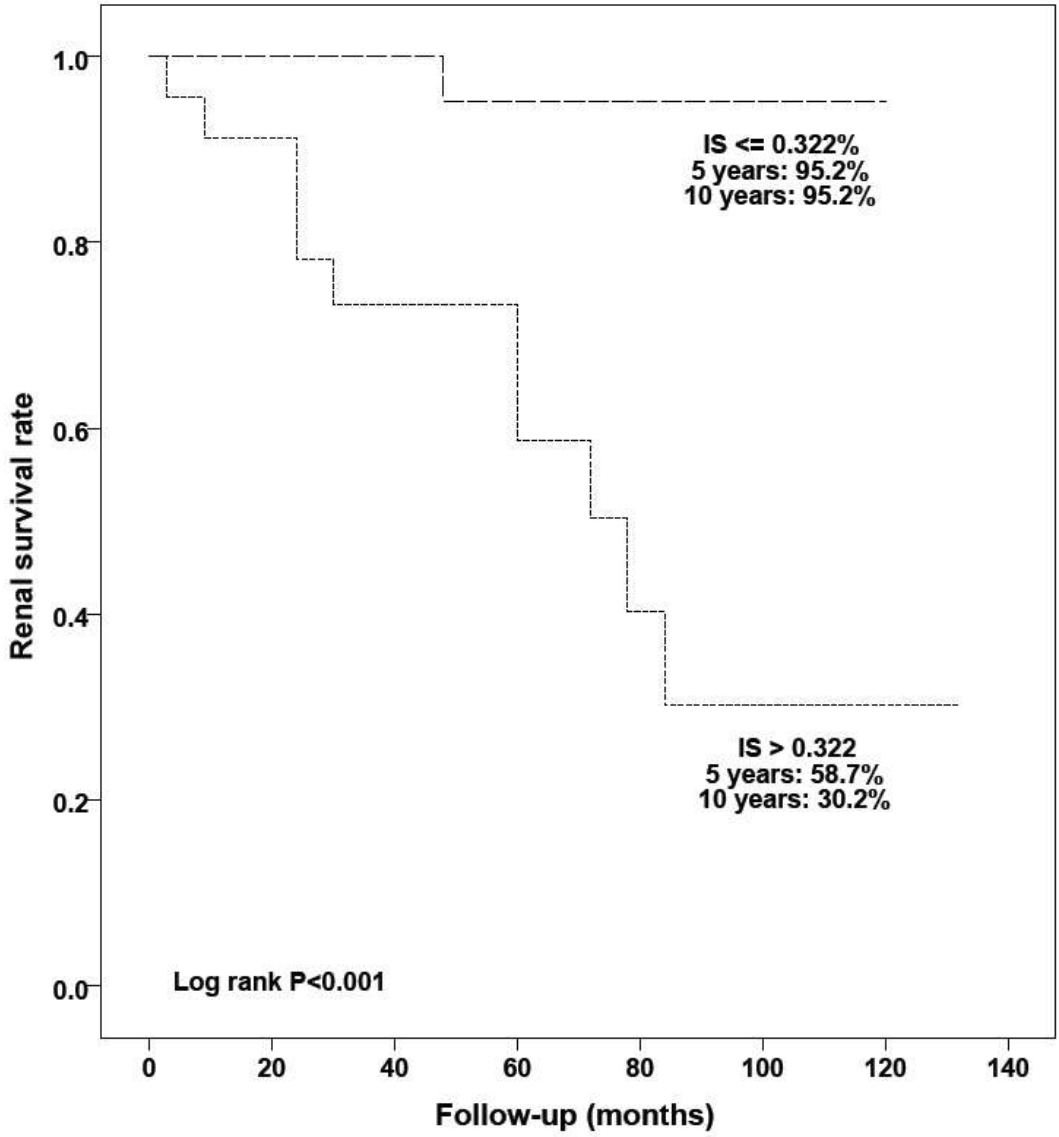

Figure 4. Renal and patient survival rate by Kaplan-Meier survival analysis and log-rank test of patients in the groups with injury score (IS) >0.322 or <=0.322.