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Measurement of Glomerular Area in Primary Glomerular Diseases With a Digital Pathology Software

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Abstract

Objective: Kidney biopsy is essential to evaluate the activity of underlying kidney disease and the risk factors related to kidney disease progression. Glomerular morphometry is a simple but uncommonly used qualitative predictor of long-term kidney function. But it is mysterious if glomerular morphometry changes due to underlying glomerulonephritis. Measurement quantification is also necessary for reliable interpretation. We aimed to evaluate glomerular morphometry with glomerular area measurements quantitatively with digitally scanned slides in the primary glomerular diseases.

Methods: We retrospectively analyzed 198 patients with nephrotic syndrome that underwent kidney biopsy between 2017 and 2019. 48 patients enrolled into the study after the exclusion criteria. We measured the glomerular area as maximum, minimum, and mean areas, using image analysis software.

Results: Forty-eight kidney biopsies were analyzed retrospectively under three primary glomerular disease groups as IgA nephropathy (37.5%), focal segmental glomerulosclerosis (29.2%), and membranous glomerulonephritis (33.3%). The glomerular area measured as maximum, minimum, and mean, were similar between 3 groups. The maximum glomerular area was negatively correlated with serum albumin and positively correlated with 24hr proteinuria (r= -0.592, p=0.044, and r=0.531, p=0.022) at the time of diagnosis. At the end of one year, maximum glomerular area was positively correlated with serum creatinine (r=0.385, p=0.019) and negatively correlated with eGFR (r= - 0.493, p=0.043).

Conclusions: At the time of diagnosis of glomerular diseases, maximum glomerular area measurement during the pathological assessment might be an additional marker to estimate the ultrastructure of the kidney and the clinical course of the disease.

Keywords: glomerular, area, proteinuria, morphometry, primary glomerulonephritis

Primer Glomerülonefritlerde Glomerül Alanın Dijital Patoloji Yazılımı ile Değerlendirilmesi

Öz

Giriş: Böbrek biyopsisi, altta yatan böbrek hastalığını ve progresyona sebep olabilecek risk faktörlerini belirlemek için gereklidir. Glomerüler morfometri basit ancak kullanılmayan, uzun dönem böbrek fonksiyonları hakkında nitel bilgi veren bir yöntemdir. Ancak altta yatan glomerülonefrit tipine göre glomerüler morfometrinin değişip değişmediği bilinmemektedir. Güvenilir değerlendirme için de ölçümlerin standart olması gereklidir. Biz bu çalışmada, primer glomerüler hastalıklarda glomerül morfometresini dijital ortamda, standart bir şekilde, glomerüler alan ölçerek değerlendirmeyi hedefledik.

Yöntemler: 2017-2019 yılları arasında nefrotik sendrom tanısı ile böbrek biyopsisi yapılmış 198 hasta restrospektif olarak değerlendirildi. Dışlama kriterleri sonrasında 48 hasta çalışmaya dahil edildi. Glomerül alanı, maksimum, minimum ve ortalama alan ölçümü olarak bilgisayar programı kullanılarak tüm seri kesitlerde incelendi.

Bulgular: Kırksekiz böbrek biyopsinde, % 37,5 hastada IgA nefropatisi, % 33,3 memranöz glomerulonefrit, % 29,2 hastada fokal segmental glomeruloskleroz tespit edildi. Üç grup arasında maksimum, minimum ve ortalama glomerül alanları benzerdi. Maksimum glomerül alanı tanı anındaki serum albumin ile negatif, 24 saatlik idrar protein miktarı ile pozitif korele bulundu (r= - 0,592, p=0,044 ve r=0,531, p=0,022). Tedavi sonrası birinci yılda, maksimum glomerül alanı serum kreatinin ile pozitif (r=0.385, p=0.019), GFR ile negatif korele bulundu (r=0,493, p=0,043).

Sonuç: Glomerüler hastalıklarda tanı anındaki patolojik değerlendirmede maksimum glomerül alan ölçülmesi, böbreğin altyapısı ve hastalığın klinik gidişatı hakkında tahminde bulunmak için ek belirteç olabilir.

Anahtar kelimeler: glomerül alanı, proteinüri, glomerül morfometri, primer glomerülonefrit, sonlanım.

INTRODUCTION

A kidney biopsy in patients with suspected glomerular disease is essential to categorize the underlying disease and predict the clinical course[1]. Histologic assessment and classification systems have a role in predicting future events, including response to immunosuppressive treatments[2]. Classification systems mainly depend on parameters like glomerular lesions, tubular atrophy, and interstitial fibrosis. Morphometric assessment of glomeruli (as glomerular area or volume) is not commonly used in this evaluation process. Glomerular area (GA) is correlated with various aspects of kidney function[3,4]. In the literature, there are studies related to GA and disease course with IgA nephropathy (IgAN)[5], idiopathic membranous nephropathy (IMN)[6], focal segmental glomerulosclerosis (FSGS)[7], and diabetes mellitus (DM)[8]. But these studies are mainly related to the pediatric population or analyzed the GA with unstandardized methods. In this study, we measured GA with a digital pathology protocol in adult patients in serial sections of the biopsy specimen. The primary objective of the study was to analyze the GA under glomerular disease type with a quantitative method. The secondary goal was to investigate the correlation between GA, clinical course, and treatment response.

METHODS

Patient Selection

Patients with nephrotic syndrome (presence of protein excretion greater than 3.5 g/24 hours, hypoalbuminemia (less than 3 g/dl), and peripheral edema)[8] that underwent kidney biopsies at the Ankara Bilkent City Hospital between 2017-2019 years were analyzed retrospectively. 198 patient records were reevaluated. Our patients were between 18-65 years old. Patients with DM and/or coronary heart disease (CHD) were not included in the study. Any patients with a moderately impaired renal function (estimated glomerular filtration rate (eGFR) <60 mL/min/1.73m2) at the time of the biopsy and those whose renal tissue specimens contained <8 glomeruli (globally

sclerotic glomeruli not included) were carefully excluded. eGFR was calculated with CKD-EPI equation[9]. Kidney biopsies from patients with FSGS and membranous nephropathy (MN) but who probably have a secondary form (lupus nephritis, obesity, infection, or malignancy-related) were excluded. At the end, 48 patients were enrolled in the study.

Patients' demographical and clinical data were obtained from the hospital's electronic records. At the time of diagnosis, body mass index (BMI), body surface area (BSA), serum creatinine (sCr), serum albumin, and 24-hour urine protein levels are assed. During follow-up period, 1st-year sCr, serum albumin, and 24-hour urine protein levels are analyzed to estimate the treatment response. Complete remission defined as proteinuria <0.3g/24 hours (uPCR <300 mg/g (<30 mg/mmol)). Partial remission defined as proteinuria >0.3 but <3.5 g/24 hours or a decrease in proteinuria by at least 50% from the initial value and <3.5 g per 24 hours. However, specific remission criteria due to underlying glomerulonephritis (GN) patterns are also carefully evaluated[10]. Supportive and immunosuppressive management of underlying glomerular diseases were assessed case-by-case on the route of recommended options of KDIGO[10].

Pathological Analysis

All kidney tissue specimens were obtained by performing a percutaneous needle biopsy. In all cases, the diagnosis of primary GN was based on characteristic findings by light microscopy (sections stained with hematoxylin and eosin, Masson-Trichrome, Jones' silver impregnation, and periodic acid-Schiff) and immunofluorescence using standard protocols. For morphometry analysis, hematoxylin and eosin-stained specimens of selected patients according to exclusion criteria were scanned with the Leica Aperio CS2 image capture device. Under 20x magnification, digital slides were created. Virapath-2.1 (Virasoft Softare Inc.,

İstanbul, Turkey) software was used to transfer digital slides into the computer. The same pathologist analyzed the digital slides and only non-sclerotic glomeruli were examined. The quantitative measurement of the GA was done with the software. The total GA (from the outline of Bowman's capsule) was measured under the 10x magnifications in the following sections. All sections were carefully examined; all glomeruli were measured with the program separately. Maximum GA, minimum GA, and mean GA, according to the number of glomeruli, calculated automatically by the same software (Figure-1).
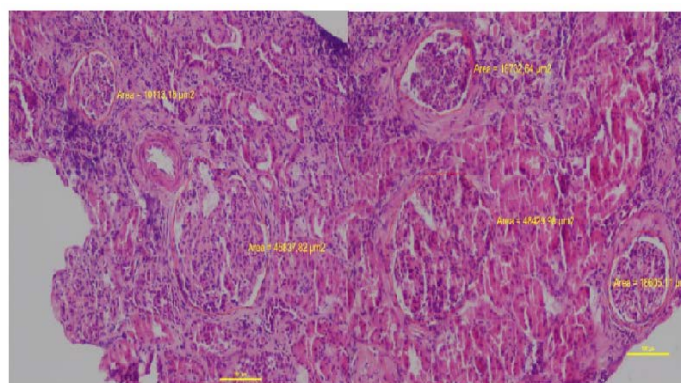

Figure 1: Measurement of glomerular area in different sections.

Statistical Analysis

The Kruskal-Wallis test and post-hoc analysis with the Tukey-Cramer test were used to analyze differences between groups. The Mann-Whitney U test was used where appropriate. Correlation coefficients were calculated using Spearmen's method. To find the factors affecting the treatment response (as no remission and partial remission/complete remission) logistic regression was used. Results were accepted as statistically significant if $p < 0.05$. All the data were analyzed using statistical software package (SPSS forWindows, Version 25.0, SPSS, USA).

Ethical issues

This study was approved by the institution's ethics committee and was carried out under the

Declaration of Helsinki (Committee decision no: E1/098/2019; 24.10.2019). All participants provided informed consent for the use of excess renal biopsy tissue available after diagnostic examination for research purposes.

RESULTS

Forty-eight kidney biopsies were analyzed retrospectively due to inclusion criteria. Biopsies are classified in three groups as IgAN; n=18 (37.5%), FSGS; n=14 (29.2%), Membranous GN; n=16 (33.3%). There was not any difference between groups for patients' age, BMI, and BSA. There was no gender difference between the groups. For all study groups, 15 (31%) patients had hypertension (HT), 8 (17%) of them in the IgAN, 5 (10%) of them in the FSGS, and 2 (4%) of them in the MGN groups. As immunosuppressive treatment, 6 (12.5 %) patients had cyclosporine, 4 (8.3 %) had cyclophosphamide and 2 (4.16 %) had tacrolimus. In laboratory measurements, for all patients (n=48), mean sCr was 0.88± 0.39 mg/dl, eGFR 97.0± 24.5 ml/min/1.73m2, serum albumin 3.43± 0.94 g/dl, and 24hr proteinuria 4735 ± 1432 mg/day, at the time of diagnosis. At the end of first year mean sCr was 1.31± 0.92 mg/dl, eGFR 93.8± 31.8 ml/min/1.73m2, serum albumin 4.10± 0.92 g/dl, and 24hr proteinuria 1174 ± 817 mg/day. At the end of 1st-year, 14 (29%) had complete remission, 23 (48%) had partial remission, and 11 (23%) had no remission.

According to underlying glomerular disease, at the time of diagnosis, serum albumin was lower, and 24h urine protein was higher in MGN group regarding the IgAN group (p=0.012, p=0.019). In 1st-year assessment, sCr and 24h urine protein was higher in the FSGS group to the IgAN group (p=0.004, p=0.031). The GA measured as maximum, minimum, and mean, were similar between groups (p=0.179, p=0.370, p=0.632). When we analyzed glomerular morphometry, whether patients had HT (15/48) or not (33/48), they were

similar, respectively (p=0.627, p=0.460, p=0.185) too. Demographic data and laboratory results are summarized in Table I.

Table I: Population characteristics and laboratory measurements according to underlying glomerular diseases

	IgAN N=18 (37.5%)	FSGS N=14 (29.2%)	MGN N=16 (33.3%)	p
Age (years)	45 (18-60)	42 (20-65)	40 (18-65)	0.123
Gender (F/M)	9/9	7/7	8/8	
BMI (kg/m²)	30 (20-38.7)	31.4(19.7-38.2)	26.2(18.8-38.7)	0.227
Laboratory Data				
sCr (mg/dl)	0.9(0.5-1.4)	0.8(0.5-1.2)	0.7(0.5-1.03)	0.076
eGFR ml/min/1.73m2	84(60-112)	100(64-129)	112(69-145)	0.122
Serum albumin (g/L)	4.0(3.2-4.7)	3.1(1.4-4.7)	3.0(1.3-4.3)	**0.012[a]**
24h proteinuria (mg/d)	2530 (840-7000)	3380 (890-15700)	5200 (2039-14300)	**0.019[b]**
Pathological Data				
Glomerular number (n)	9 (8-14)	9 (8-18)	9 (8-15)	0.406
Maximum GA (µm²) x10³	40.3(22.9-81.6)	48.9(23.1-71.4)	38.9(28-51.9)	0.179
Minimum GA (µm²) x 10³	16.7(6.0-25.6)	14.6(8.9-28)	15.2(10.2-25.6)	0.370
Mean GA (µm²) x10³	28.9(16.7-43.4)	31.1(17.1-39.9)	27.9(18.7-36.9)	0.632
Glomerular number (n)	9 (8-14)	9 (8-18)	9 (8-15)	0.406

BMI: Body mass index, BSA: Body surface area, eGFR: estimated glomerular filtration rate, FSGS: Focal segmental glomerulosclerosis, HT: Hypertension, IgAN: IgA nephropathy, MGN: Membranous glomerulonephritis, sCr: serum creatinine. Data presented as median and (min-max). a, b IgAN and MGN are statistically different in between.

In correlation analysis of all groups (n=48), only maximum GA was negatively correlated with serum albumin and positively correlated with 24hr proteinuria (r= - 0.592, p=0.044, and r=0.531, p=0.022) at the time of diagnosis. At the end of one year, maximum GA was positively correlated with serum creatinine (r=0.385, p=0.019) and negatively correlated with eGFR (r= - 0.493, p=0.043). But not correlated with the serum albumin and proteinuria at the end of 1st year. None of laboratory or glomerular measurement type was statistically significant to explain remission state (as no remission and partial remission /complete remission) at the end of 1st year in the logistic regression analysis.

DISCUSSION

The present study has two main findings. First, according to underlying glomerular disease, the maximum GA did not differ. Second, the maximum GA was correlated with hypoalbuminemia and proteinuria at the time of diagnosis, and serum creatinine and eGFR at the end of the 1st year. We carefully excluded patients with secondary GN, DM, CAD, or decreased eGFR that would end with glomerular hemodynamic and structural changes. None of our patients were obese. Median age, female/male ratio, BMI, and BSA were similar regarding the underlying primary disease as IgAN, FSGS, and MGN.

It is known that glomerular enlargement is related to increased metabolic demands or decreased renal mass. But the mechanism under the expansion of the glomerulus is not that simple. Glomerular cell injury type also affects the glomerular morphology[7]. In an experimental study of five-sixths of nephrectomized rats with proteinuria, She et al. found that glomerular hypertrophy is related to mesangial cell proliferation and progressive increase of the mesangial matrix. It was a pathological process rather than a compensatory mechanism[11]. In humans, studies

are generally related to obesity-related glomerular enlargement. Kambham et al. reported in 2011 that obesity-related glomerulomegaly with or without FSGS, associated with microalbuminuria beyond the increased metabolic demands[12]. Wu et al. analyzed the expression of inflammatory genes- in the glomeruli- in obese patients with proteinuria. And they found that glomerular enlargement was probably related to chronic inflammation[13]. Glomerular injury type is also associated with the mechanism of enlarged GA[14,15], and the progression of injury, in turn, affects the glomerular size[16]. Increased glomerular size in DM as a result of compensatory hypertrophy[8], and decreased size in the course of senescence[17] are known facts. But in the course of proteinuria, we do not know whether the underlying disease or proteinuria itself directly affects the ultrastructure. In our study, maximum GA, minimum GA, and mean GA were similar for the primary glomerular disease type. But in correlation analysis, maximum GA was the only measurement related to low serum albumin and high proteinuria. Maximum GA, as an indicator of enlarged glomerulus, might represent ultrastructure problems of kidney like podocyte injury. Or heavy proteinuria itself cause glomerular enlargement at the time of diagnosis of the glomerulonephritis. None of our patients were obese that would cause glomerulus enlargement secondarily. Although our subject number is low, it is likely to speculate that glomerular size is not only affected by the injury site of primary disease but also from proteinuria itself.

In other studies of renal ablation models, it was also suggested that the glomerular enlargement precedes the development of renal scarring[18,19]. It is reported that some injured glomeruli increase in size before they sclerosed or collapsed[20]. This theory may be related to low nephron number too. Without any apparent

renal dysfunction, nephron number in healthy people may vary. And it is shown that low nephron number is associated with poor outcomes of glomerular diseases[21]. It is not clear GA represents the nephron number of the whole kidney. But as we found in our study, increased glomerular size would be a primary step toward glomerulosclerosis. And our results indirectly support the idea that GA may be used as a marker of kidney nephron capacity. The morphometry might be a predictor for renal survival in the course of primary GN.

For histological sclerosis assessment, not only counting injured glomerulus but also enlargement of glomerulus should be used as an alarming sign of bad renal outcome in the preinjury period. Unfortunately, there is not a standardized measurement method for the assessment of GA. The Weibel and Gomez method[22] or mean planar area (MPA) measurement[3] are used common methods. Weibel and Gomez estimates GA from a single histological section that cannot quantify the variation in the size of the glomerulus. MPA allows serial sectioned measurement of the tissue and identify the most significant profile for each glomerulus. But pathologists should keep in mind that examination of enlarged glomerulus concerning normal and sclerosing/collapsing glomerulus as maximum GA measurement may facilitate wrong measurement of glomerular size. In our study, measurements were based on MPA using digital pathology software. We measured all glomeruli in each section, and automatically measured the maximum and the minimum GA with the program. The mean GA was measured from all glomeruli which were examined for the same specimen. In this way, our study is unique that measures the glomerular size in very standardized way without any subjective assessment.

In long-term prognosis prediction studies, morphologic assessment of glomeruli was used to predict GFR loss, or 10-year renal function outcomes in several glomerular diseases[4]. Maximum GA was investigated as a prognostic indicator in IgAN, FSGS, and DM[23,24,8] beyond the routine classification algorithms. Kataoka et al. revealed in their study that including maximum glomerular diameter in the Oxford classification of IgAN might increase its strength in the clinical setting[25]. Tsuboi and et al. evaluated glomerular density (GD) (inversely correlated with glomerular area) in 98 IgAN patients. And they displayed that low GD is related to enhanced risk for progression although interstitial fibrosis and degree of glomerulosclerosis were not independent predictors[26]. Our findings about 1st year serum creatinine and eGFR and GA correlation coincides with these findings. And also with more reliable measurement method, the idea was strengthened.

Our study has some limitations. Low subject number in the disease groups may empower the primary disease-related glomerular enlargement and related analysis. And in the literature, it is not apparent the exact cut-off value of enlarged glomerular diameter, which would cause unstandardized analysis of glomerular morphometry.

In conclusion, in the early period, maximum GA may be a marker of worse ultrastructural problems of the kidney. Enlarged glomeruli might estimate clinical course irrespective of underlying glomerular pathology. Using digital pathology software minimizes the pathologist related subjective measurements. With a high number of subjects, this finding should be optimized.

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