Original Articles/นิพนธ์ต้นฉบับ

Extracellular Matrix and Collagen Index in Lupus Nephritis.

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Abstract

Background: Interstitial fibrosis is associated with poor long term outcome in many kidney diseases. We investigated the predictive value of extracellular matrix quantification on long term outcome in patient with lupus nephritis.

Methods: A cohort of 43 patients with lupus nephritis (LN) was followed from the time of biopsy. Kidney biopsy specimens were stained with picro-sirius red. The magnitude of fibrotic tissue was calculated by computerized image analysis. Each biopsy tissue was evaluated for different parameters: collagenous matrix index (CMI), fibrillary collagen index (FCI), activity index and chronicity index.

Result: At baseline, median serum creatinine(Cr) was 0.8 mg/dl and mean eGFR was 90.3±37.6 ml/min/1.73 m^2. Median follow up was 56.4 (range 1.2-13.2) months. Thirty-three patients achieved remission (22 complete remission, 11 partial remission), 20 patients had decreased 25%eGFR, 6 patients had renal failure (dialysis, eGFR<15 or Cr>5) and 9 patients died. At the time of biopsy, the activity index correlated with Cr, eGFR and proteinuria. Chronicity index correlated with Cr and eGFR. CMI correlated with Cr and tended to correlate with eGFR. Low CMI predicted good long term renal outcome as assessed by 25% decreased eGFR, renal failure and death. Low activity index was associated with earlier remission. The activity index, chronicity index and FCI predicted of long term renal outcome.

Summary: A computerized system for extracellular matrix quantification of LN predicts long term renal outcome. Future studies are necessary to determine benefit.

Keywords: Lupus nephritis, sirius red, collagen; fibrosis; kidney; renal failure

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Introduction

Systemic lupus erythematosus (SLE) is common in adult Thai women.[1] The disease can involve many systems and the clinical manifestations are variable. Both disease activity and immunosuppressive agents used in therapy contribute to morbidity and mortality in SLE.[2] Kidney involvement is common and increases adverse outcomes in SLE. Lupus nephritis (LN) can encompass many forms of glomerulonephritis. A renal biopsy is used to classify patients according to class (e.g. WHO) and to evaluate the degree of acute inflammation as well as chronic changes such as tubular atrophy and tubulointerstitial scarring. Patients in the same renal histopathologic class may have different long term outcomes. Some patients have good responses to treatment, whereas others may develop a decline in renal function and progress to end stage renal failure.

The activity index and chronicity index have been shown to be useful in predicting outcomes in patients with LN in some studies. However, the benefit of activity index and chronicity index have been disputed in other studies.[3] Interstitial fibrosis, a component of chronicity index, develops as part of an irreversible scarring process due to extracellular matrix and collagen deposition.[4] Interstitial fibrosis leads to progressive worsening of renal function and has been shown to correlate with poor clinical outcomes in many renal diseases including LN[1,5,6]

The precise quantification of interstitial fibrosis may be difficult using current histopathologic systems, in which interstitial fibrosis is scored semi-quantitatively by grid counting. Such methods are subjected to high degree of inter-observer variations and can be time consuming to perform. Recently, computerized image analysis of interstitial volume and collagen staining has been performed using sirius red staining.[7] Sirius red has been used as a specific method for detection of collagen in tissue sections. The dye, which intercalates into the tertiary groove of collagens I and III, imparts a pink stain to most tissues when observed under white light and is birefringent when observed under polarized light. The extent of interstitial fibrosis as quantitated by computerized imaging of sirius red stained kidney biopsy has been shown to be associated with outcome in the renal allograft.[7,8] In this study, we hypothesized that the assessment of interstitial fibrosis by picrosirius red stain (collagen stain) would predict long term outcomes in lupus nephritis.

Materials and Methods

Patient population and preparation of biopsies

Patients who had a biopsy proven diagnosis of LN from August 2000- February 2002 at Ramathibodi Hospital were evaluated. Patients with complete clinical history and kidney biopsy tissue available were selected. Kidney biopsy tissues were fixed in Zenker and embedded in paraffin. Paraffin embedded tissue was sectioned at 3-4 um thickness. Deparaffined slides were stained by hemoatoxylin and eosin, PAS according to standard protocols. WHO classification, activity and chronicity scores were assigned by a trained nephropathologist without knowledge of patients' clinical details. This study was approved by the Ethical Committees of Ramathibodi Hospital (ID 085/2006).

Preparation of picrosirius red stain

In addition to standard histochemistry staining, the kidney section was stained with picrosirius red using published protocols.[7] Picrosirius red (0.1%) was prepared from 1% Sirius red solution and saturated picric acid. Slides were baked at 50°C for 2 hours, soaked in xylene and subsequently in acetone for 24 hours each. Slides were then deparaffinized in xylene for 5 min, hydrated in ethanol (100%, 95%, 80%, 60%) for 2 min, washed and stained in picrosirius red overnight. Zenker stain was removed by soaking in 1% iodine in 80% ethanol for 2 min and

then 5% sodium thiosulfate for 2 min after the 80% ethanol step. The next morning, slides were put in 0.01N hydrochloric acid for 2 min, and pidly dehydrated through graded ethanol startin at 70% to 100%, then to xylene, and finally were cover slipped with Permount.

Imaged acquisition

Images of the whole kidney biopsy section were randomly photographed with digital camera (DS camera Control Unit DS-L2, Nikon) attached to Nikon Optishot 2 microscope. Ten images (x 40 magnification) were obtained for each slide section by working from one end of the tissue to another in a serpentine manner. The kidney cortex was imaged in bright field and in double polarized light field. A background correction was performed in real time to adjust for minor irregularities in illumination of the microscopic field. The light source and the condenser were used with fixed setting for all images. Medulla, capsule, glomerular portions and blood vessels were avoided when acquiring the images.

Image analysis

Image analysis was analyzed by free software (Image J1.30; National Institutes of Health, Bethesda, MD, USA). Images were converted from red, green, and blue (RGB) color (as captured) to gray scale RGB stack, then differentiated into three gray scale images representing red, green and blue. The gray scale is 0 (black) to 255 (white). For analysis, the green-gray scale images were used in bright field picture and the red-gray scale images were used in polarized light picture. The parameter areas were measured by threshold graph. The spaces for measurements were total area, intraluminal area, interstitial area, polarized positive area. The total cortical area was the area of the field minus the intraluminal area.

Parameters were defined as follows. Collagenous matrix index (CMI) was the percentage interstitial area (stained deep red) under white light of the total cortical area. Fibrillary collagen index (FCI) was the percentage area of cortical area that showed positive staining under polarized light.

Collection of clinical data

Patients were managed according to individual physician's discretion. Clinical, laboratory notes and electronic databases were reviewed for data at the time of the biopsy and during follow up. Estimated glomerular filtration rate (eGFR) was calculated using MDRD equation[9]

End points

The following end points were chosen and defined, based on a recent consensus meeting[10]: complete remission (proteinuria < 0.2 g/24 hours or urine protein/creatinine ratio (UPCR) < 0.2, no active urine sediment, and plasma creatinine no worsen from previous baseline (25% decline in eGFR), partial remission (0.2 g/24 hours < proteinuria < 2 g/24 hours or 0.2< UPCR <2 and plasma creatinine no worsen from previous baseline (25% decline in eGFR), relapse (proteinuria >2 g/24 hours or UPCR > 2 after remission), declined eGFR > 25%, end stage renal disease ESRD (dialysis or eGFR < 15 ml/min/1.73 m^2) and death.

Statistical analysis

Data were summarized by the means (normal distribution), median (not in normal distribution) and percentages. The four main parameters were assessed for correlation with eGFR and 24-hour proteinuria at the time of biopsy and disease outcome at the time of follow up. Spearman's rank correlation test was used to test for correlation between clinical disease outcomes and indices. Survival methods were used to test the prognostic values of the biopsy indexes (CMI, FCI, activity index and chronicity index) in predicting the outcomes by the log-rank statistic. As

an adjunct to analyzing the original index values, patients were dichotomized into high and low index and Kaplan-Meier plots were presented for illustration. The activity and chronicity index split by median. High CMI or FCI index defied as the score ≥ upper quartile. All analysis was performed using the standard software STATA version 9.0.

Results

Patient characteristics and outcome

Forty -three SLE patients were included in this study. The patients were predominantly young adult women (Table 1). At the time of biopsy, the median Cr was 0.8 mg/dl (0.5-9.2), proteinuria was 2.5 g/day (0.3-20) and the mean +/- SD eGFR was 90.3 ± 37.6 ml/min/1.73 m^2. Most patients (67%) had active urine sediment at presentation. The majority of patients (86%) had previous corticosteroid or immunosuppressive treatment before biopsy. Nearly half the subjects (47%) had WHO class IV, and 12 patients (28%) had class V.

Patients were followed-up for a minimum of 1.2 months and maximum of 73.2 months (median 56.4 months). Thirty-three patients (76%) achieved remission (22 complete remission, 11 partial remission), 16 had relapsed, 20 had decreased 25% eGFR, 6 had renal failure (dialysis, eGFR <15 or Cr >5) and 9 (21%) died.

Correlation of histological variables at baseline biopsy

At the time of biopsy, CMI correlated with Cr (r = 0.45, P = 0.003) and tended to correlated inversely with eGFR (r = -0.29, P = 0.06) (Figure 1). But the degree of proteinuria had no correlation with CMI (r = -0.56, P = NS). On the other hand, FCI had no correlations with Cr (r = 0.02, P = NS), eGFR (r = 0.02, P = NS), or proteinuria; (r = -0.27, P = NS).

An activity index correlated with Cr (r = 0.43, P = 0.005), eGFR (r = -0.42, P = 0.007) and proteinuria (r = 0.36, P = 0.03) (Figure 2). Chronicity index correlated with Cr (r= 0.38, P = 0.014) and inversely with eGFR (r = -0.36 P = 0.019) (Figure 3).

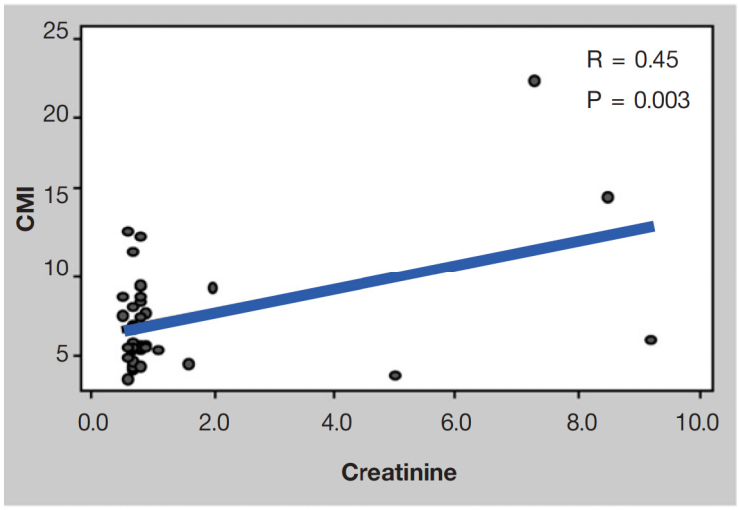
Correlation of histological variables at follow up period

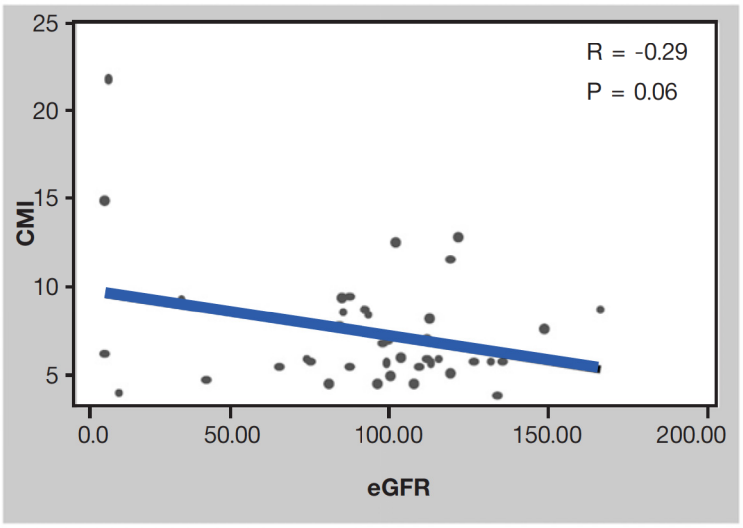
Based on upper quartiles of distribution, low CMI was defined as CMI < 8.7 and the high CMI was CMI ≥ 8.7. The low FCI was FCI < 4.5 and the high FCI was FCI ≥ 4.5. Based on median scores, the low activity index was activity index ≤ 3 and high activity index was activity index ≤ 3. The low chronicity index was chronicity score = 0 and high chronicity index was chronicity score > 0.

Table 1. Baseline Characteristics and biopsy data for patient group

Baseline Characteristics	N = 43
Age	32 ± 10.6
Sex (Women/Men)	41/2
Systolic BP (mmHg)	130.9 ± 13.6
Diastolic BP (mmHg)	81.6 ± 10.2
Weight (kg)	54.6 ± 10.9
Serum albumin (g/l)	29.9 ± 7.9
Creatinine (mg/dl)	0.8 (0.5-9.2)
eGFR (ml/min/1.73 m^2)	90.3 ± 37.6
Urine protein (g/day)	2.5 (0.3-20)
Active urine sediment (%)	67
Treatment before biopsy (%)	86
Lupus classification (WHO)	
II	23%
III	0%
IV	47%
V	28%
VI	2%
Activity index	3 (0-16)
Chronicity index	0 (0-5)
CMI	7.4 ± 3.4
FCI	3.7 ± 1.3

Data is shown as mean ± SD or median (range) or percent.
eGFR = estimated glomerular filtration rate,
CMI = collagen matrix index, FCI = fibrillary index





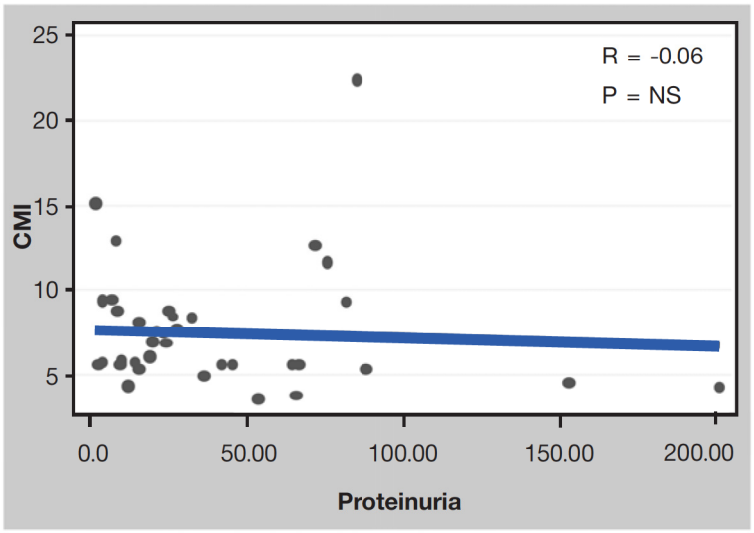
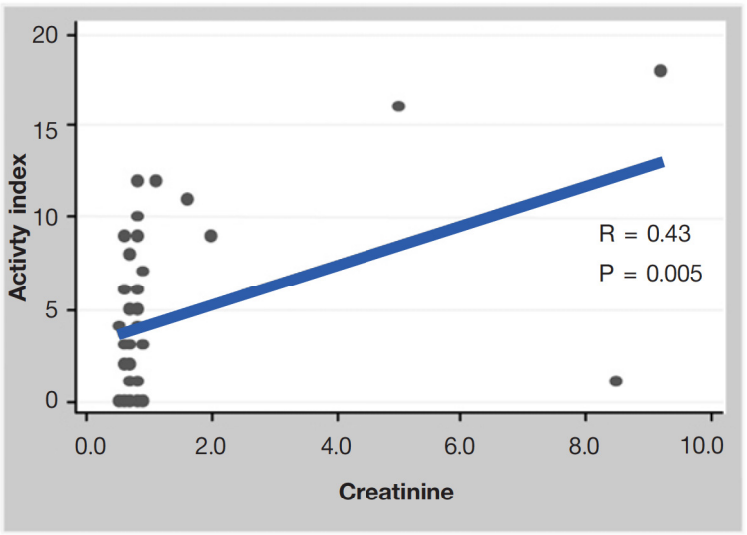
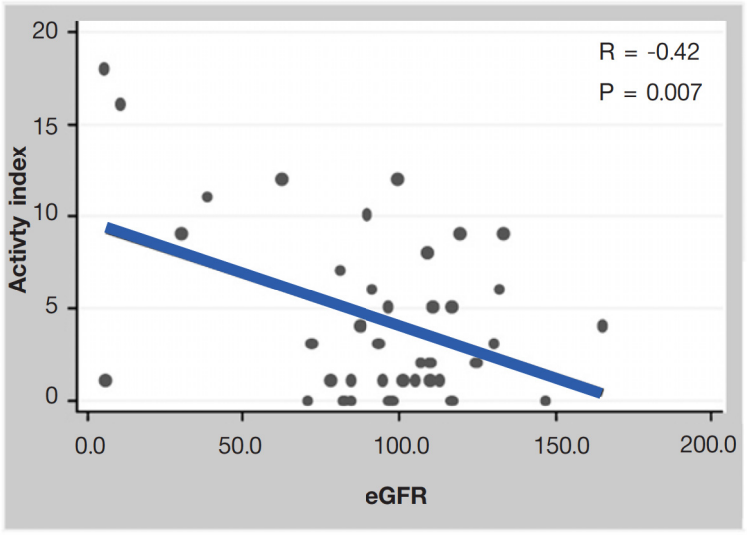


Fig.1 *Correlation of collagenous matrix index (CMI) with clinical parameter (at time of biopsy)*





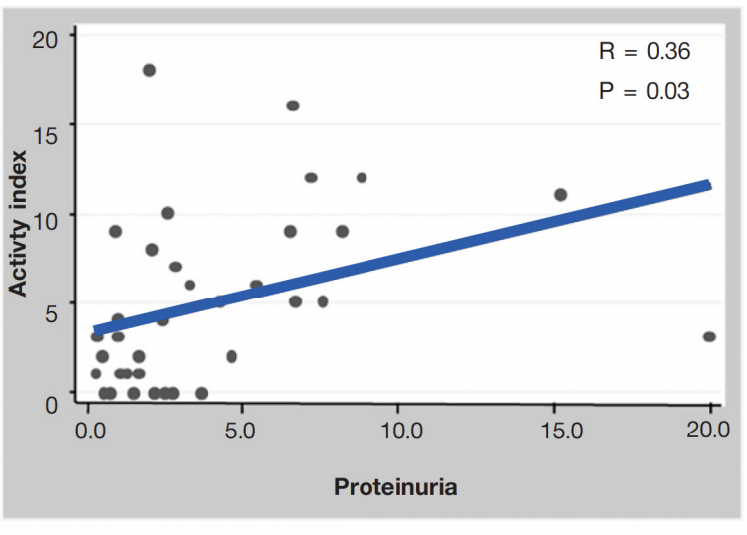
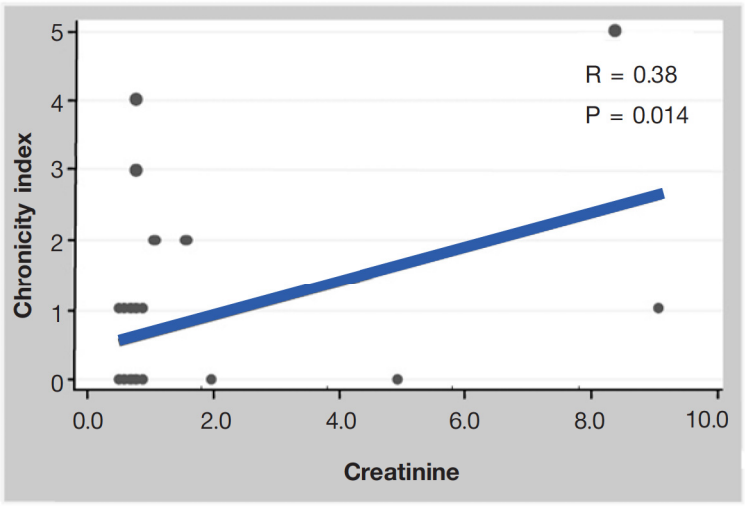
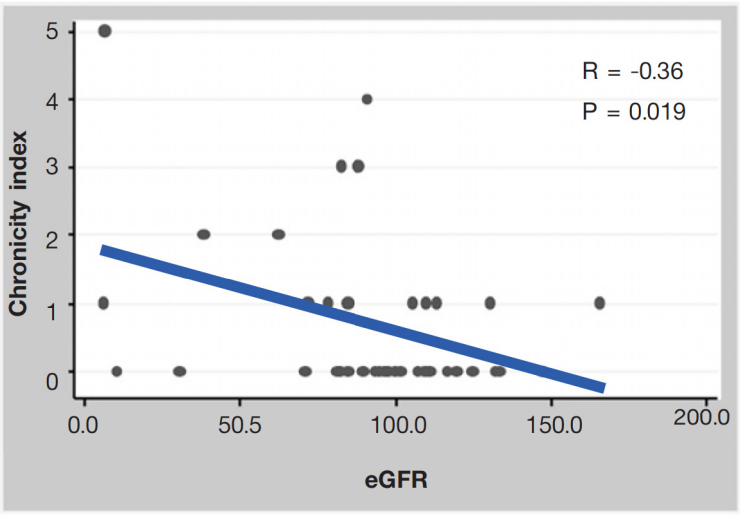


Fig.2 *Correlation of activity index with clinical parameters (at time of biopsy)*





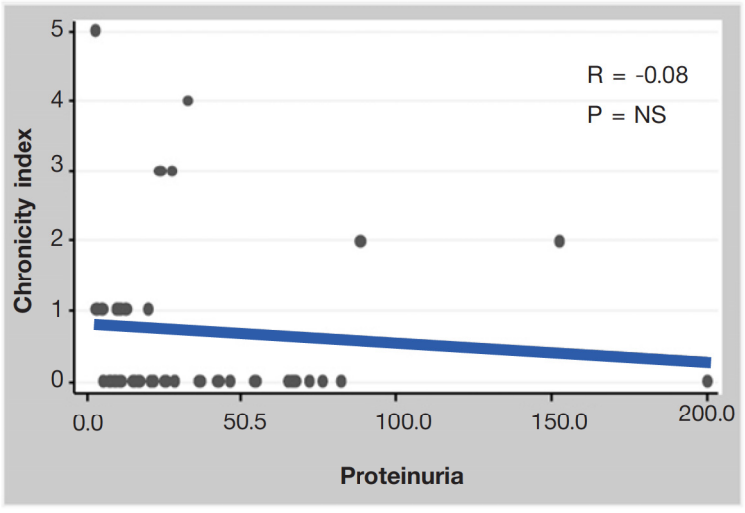


Fig.3 *Correlation of chronicity index with clinical parameters (at time of biopsy)*

Remission

Using survival analysis, activity index predicted remission (P = 0.028). Low activity index had early remission (median time remission = 7.03 months in low index, 17.5 months in high index). No parameter predicted relapse.

Decreased 25% eGFR

Only CMI predicted 25% *decline eGFR* (P = 0.005). (Figure 4). FCI tended to correlate with 25% *declined eGFR* (P = 0.067).

Renal failure and death

Using survival analysis, CMI significantly predicted renal failure (*P* = 0.019), and death (*P* = 0.0002) (Figures 5 and Figures 6). The other parameters had no significant correlation with renal survival and death.

Discussion

In this study, we demonstrated that extracellular matrix deposition measured by digital quantification of the positive picrosirius stained area correlated with long term renal outcome and mortality in patients with lupus nephritis. Low activity index predicted early remission, but did not predict renal failure.

Sirius red is a histochemical stain that has been used for nearly 30 years. The dye molecule intercalates into the tertiary groove in the structure of collagen types I and III and imparts a pink stain to most tissues when observed under white light. Our study shows the practicability and utility of simple computer-aided morphometry to help renal biopsy evaluation in lupus nephritis. All indices measured involve a high contrast signal (stain vs. no stain) and has good reproducibility on different days and among different observers.[7] In animal models, computerized image analysis of picro-sirius red staining has been used to accurately quantitate both the age of fibrotic lesions and their extent.[12] Picrosirius red has also been used to quantitate the reduction in interstitial fibrosis after therapeutic intervention in the 5/6 nephrectomy rat model.[10]

In human studies, picrosirius red staining has been used to quantitate fibrotic changes in pediatric liver transplant biopsies[13] and the response to interferon alpha 2B therapy in patients with chronic non-A, non-B hepatitis.[8] In this latter study, picro-

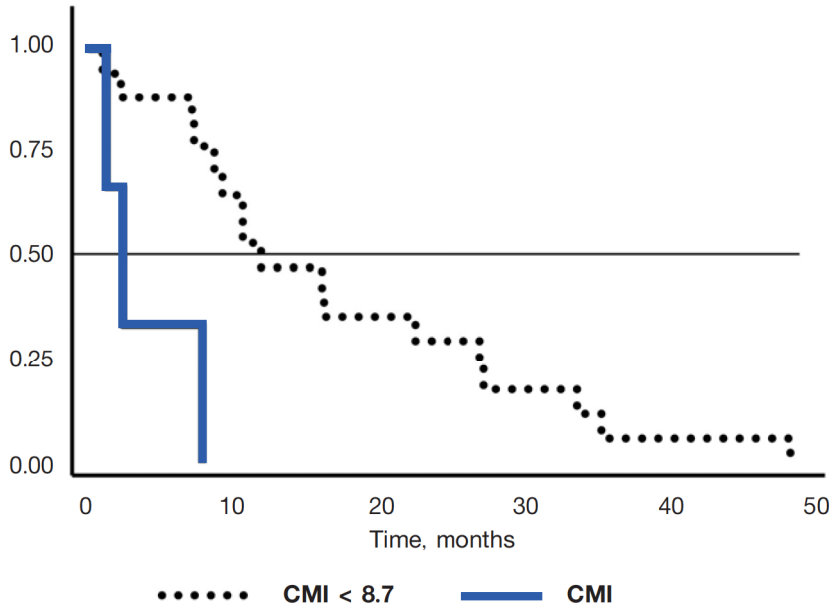


Fig.4 Survival plot of the proportion without decrease 25% eGFR according to collagenous matrix index (CMI) status

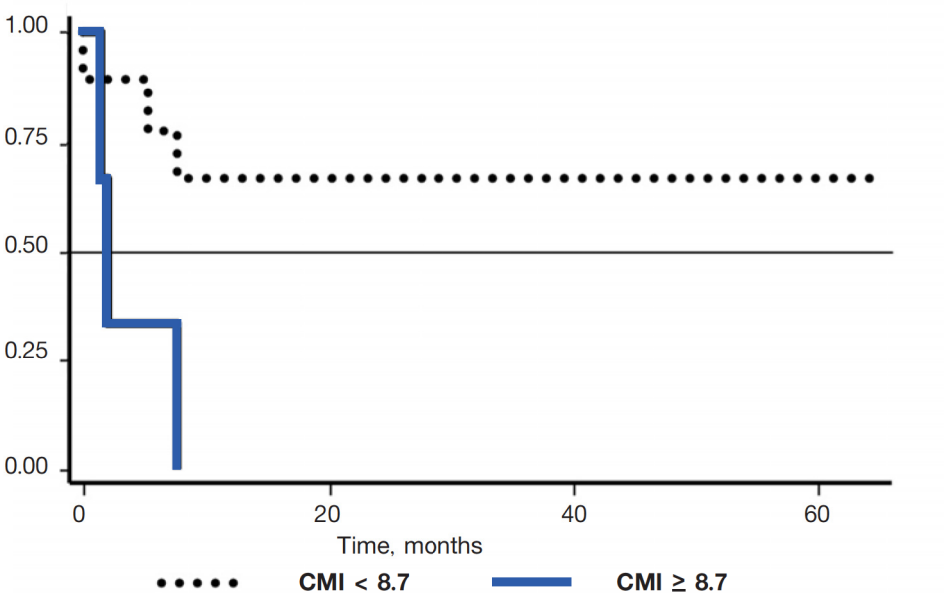


Fig.5 *Survival plot of the proportion without reaching renal failure according to collagenous matrix index (CMI) status*

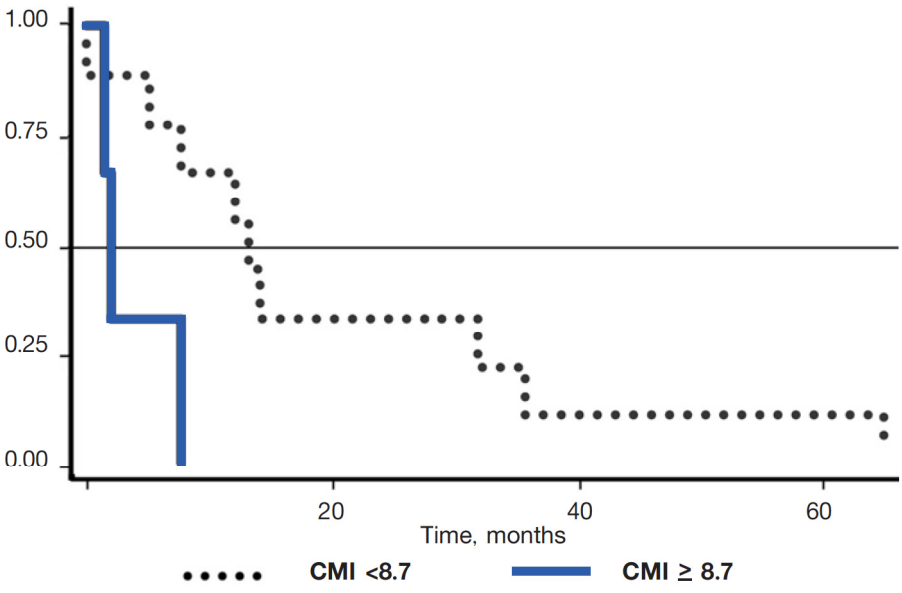


Fig.6 *Survival plot of the proportion survive according to collagenous matrix index (CMI)*

sirius red staining was shown to be highly correlated with total liver collagen as determined by hydroxyproline assay. In renal biopsies, picrosirius red staining has been quantitated by computerized image analysis, has been shown to correlate not only with the interstitial volume fraction of the cortex as measured by the more laborious point counting method,[14] but also with the GFR at the time of biopsy.[15]

In our study, we used four main parameters (activity index, chronicity index, CMI, FCI) to correlate clinical outcome. Only the collagen matrix index strongly correlated with renal outcome and death. The activity index was the good predictor initial clinical disease severity, but not long term outcome. Only one previous study has examined the correlations of collagen matrix deposition as determined by picrosirius red staining and outcome in lupus nephritis.[16] Similar to our study, Hunter MG et al also demonstrated that high matrix index predicted adverse renal outcome in Caucasian subjects with lupus nephritis.

A major advantage of this study is that subjects had long follow up therefore the long term outcome to be assessed. Limitations of this study include the relatively limited number of biopsies available for study. Secondly, since this is retrospective study, some clinical data may be incomplete. At present, the optimal cut point for each parameter to predict outcome is unknown. In the future, it would be advantageous to test this method in another, perhaps larger cohort of lupus patients.

Conclusions

A computerized system for extracellular matrix quantification of LN predicts long term renal outcome. At baseline, activity index, chronicity index correlated with eGFR. At long term follow up, low activity index predicted earlier remission and low CMI predicted good long term renal function. Future studies are necessary to determine the benefit of these new histomorphometric studies.

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Original Articles/นิพนธ์ต้นฉบับ

Extracellular Matrix and Collagen Index in Lupus Nephritis

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บทคัดย่อ

วัตถุประสงค์: จากการศึกษาในอดีตหลายรายงานพบว่าการเกิดพังผืดในไตจะสัมพันธ์กับการดำเนินโรคที่ไม่ดีในโรคไตหลายชนิดและน่าจะมีผลทำให้ผู้ป่วยเข้าสู่ภาวะไตวายระยะสุดท้ายได้มากกว่าผู้ที่มีพังผืดในไตน้อยกว่าในผู้ป่วยกลุ่มโรคเดียวกัน ซึ่งในการศึกษานี้ผู้วิจัยจึงได้ทำการศึกษาเพื่อดูผลของการวัดปริมาณ extracellular matrix ซึ่งสัมพันธ์กับการเกิดพังผืด เพื่อใช้ในการพยากรณ์การดำเนินโรคระยะยาวในผู้ป่วย Lupus nephritis (LN)

วิธีการวิจัย: เป็นการศึกษาย้อนหลังโดยเก็บข้อมูลของผู้ป่วย LN 43 รายที่ได้รับการเจาะชิ้นเนื้อไตในช่วงเดือนสิงหาคม ปี พ.ศ. 2543-กุมภาพันธ์ ปี พ.ศ. 2545 ตั้งแต่วันที่มีการเจาะชิ้นเนื้อและติดตามผลการดำเนินโรคต่อเนื่อง สำหรับชิ้นเนื้อที่ได้จะถูกนำมาย้อมพิเศษ picrosirius red และนำมาถ่ายภาพและวัดหาค่าพารามิเตอร์ต่างๆ โดยใช้โปรแกรมทางคอมพิวเตอร์และคำนวนหาค่าดังนี้ collagenous matrix index (CMI), fibrillary collagen index (FCI), activity index and chronicity index

ผลการศึกษา: ที่เริ่มต้นการศึกษาพบว่าผู้ป่วยมีค่าเฉลี่ยของ serum creatinine (Cr) และการทำงานของไต (eGFR) 0.8 มก/ดล และ 90.3 มล/นาที/1.73 $ม^2$ ตามลำดับ ได้รับการติดตามการรักษาเฉลี่ย 56.4 เดือน เมื่อติดตามการรักษาพบว่ามีผู้ป่วยเข้าสู่ remission ทั้งหมด 33 ราย แบ่งเป็น complete remission 22 ราย และ partial remission 11 ราย ผู้ป่วย 20 รายมีค่าการทำงานของไตลดลงร้อยละ 25, 6 รายเข้าสู่ภาวะไตวาย (ได้รับการล้างไต, eGFR<15 หรือ Cr>5) และ 9 รายเสียชีวิต ที่เวลาตั้งต้นพบว่า activity index สัมพันธ์กับค่า Cr, ค่าการทำงานของไต และปริมาณโปรตีนในปัสสาวะ chronicity index สัมพันธ์กับค่า Cr และค่าการทำงานของไต CMI สัมพันธ์กับค่า Cr ที่ตั้งต้น เมื่อติดตามผลระยะยาวพบว่า การเข้าสู่ remission จะสัมพันธ์กับค่า activity index สำหรับผู้ป่วยที่มี CMI index ต่ำ พบว่ามีค่าการทำงานของไตลดลงร้อยละ 25, การเกิดไตวายและเสียชีวิตน้อยกว่าผู้ป่วยที่มีค่า CMI index สูง แต่ค่า activity index, chronicity index และ FCI ไม่พบมีความสัมพันธ์กับการดำเนินของโรคในระยะยาว

สรุปผลการศึกษา: การวัดปริมาณ extracellular matrix โดยเทคนิคทางคอมพิวเตอร์น่าจะสามารถใช้ในการพยากรณ์การดำเนินของโรคในผู้ป่วย LN ได้ แต่ต้องการข้อมูลการศึกษาที่มากขึ้นในอนาคตเพื่อยืนยันถึงประโยชน์และความคุ้มค่าต่อไป