DETECTION OF KIDNEY INJURY BY USING THE CORRELATION OF PERIOSTIN AND RENAL PATHOLOGY IN PATIENTS WITH LUPUS NEPHRITIS AND IgA NEPHROPATHY

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การตรวจหาการบาดเจ็บของไตโดยใช้ความสัมพันธ์ระหว่างเพอริออสตินกับพยาธิสภาพของไตใน ผู้ป่วย โรคไตอักเสบลูปัส และผู้ป่วยโรค IgA nephropathy

นางสาวปีย์ภัทรา วันทนาศิริ

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญาเภสัชศาสตรดุษฎีบัณฑิต
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Thesis Title	DETECTION OF KIDNEY INJURY BY USING THE CORRELATION OF PERIOSTIN AND RENAL PATHOLOGY IN PATIENTS WITH LUPUS NEPHRITIS AND IgA NEPHROPATHY
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ปีย์ภัทรา วันทนาศิริ : การตรวจหาการบาดเจ็บของไตโดยใช้ความสัมพันธ์ระหว่างเพอริออสตินกับพยาธิสภาพของไตในผู้ป่วย โรคไตอักเสบลูปัส และผู้ป่วยโรค IgA nephropathy (DETECTION OF KIDNEY INJURY BY USING THE CORRELATION OF PERIOSTIN AND RENAL PATHOLOGY IN PATIENTS WITH LUPUS NEPHRITIS AND IgA NEPHROPATHY) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: ศ. ภญ. ดร. พรอนงค์ อร่ามวิทย์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: พ.ต. นพ. บัญชา สถิระพจน์, 162 หน้า.

การวิจัยนี้มีวัตถุประสงค์เพื่อตรวจหาความสัมพันธ์ระหว่างระดับเพอริออสตินในตัวอย่างชิ้นเนื้อไต ปัสสาวะ และซีรั่ม กับพยาธิสภาพของไตและค่าการทำงานของไตในผู้ป่วยโรคไตอักเสบลูปัส และผู้ป่วยโรค IgA nephropathy และตรวจหาระดับเพอริออสติน ในกลุ่มผู้ป่วยเปรียบเทียบกับกลุ่มควบคุม รวมทั้งประเมินความเป็นไปได้ในการทำนายการตอบสนองต่อการรักษาจากการตรวจวัดเพอริออสติน การศึกษานี้ดำเนินการวิจัยที่ กองอายุรกรรม โรงพยาบาลพระมงกุฎเกล้า ในช่วงเดือนเมษายน 2556 ถึงเดือนกุมภาพันธ์ 2558 โดยมีผู้ป่วยเข้าร่วมในการศึกษาจำนวน 50 ราย เป็น ผู้ป่วยโรคไตอักเสบลูปัส 37 ราย และผู้ป่วยโรค IgA nephropathy 13 ราย และ อาสาสมัครสุขภาพดีจำนวน 50 ราย สำหรับกลุ่มควบคุมชิ้นเนื้อไตทำการเก็บตัวอย่างชิ้นเนื้อไตที่ปกติจากผู้ป่วย renal cell carcinoma 5 ราย ผลการประเมินการย้อมชิ้นเนื้อไตเพื่อตรวจหาปริมาณเพอริออสติน พบว่าชิ้นเนื้อไตในกลุ่มควบคุมไม่พบการติดของเพอริออสติน ส่วนชิ้นเนื้อไตจากกลุ่มผู้ป่วยพบการติดของเพอริออสตินส่วนใหญ่ในบริเวณ tubule นอกจากนี้ ยังพบเพอริออสตินในบริเวณ periglomeruli, sclerosed glomeruli, interstitial fibrosis และ vascular fibrosis ผลการวิเคราะห์หาความสัมพันธ์ระหว่างการติดของเพอริออสตินในชิ้นเนื้อไตกับพยาธิสภาพในไต พบความสัมพันธ์อย่างมีนัยสำคัญทางสถิติระหว่างเพอริออสตินในชิ้นเนื้อไตกับความเรื้อรังของโรคไต ($p<0.05$) และยังพบความสัมพันธ์กับค่าการทำงานของไต นอกจากนี้เมื่อทำการวิเคราะห์ในกลุ่มผู้ป่วยที่มีความรุนแรง และความเรื้อรังของโรคต่ำ พบว่าผู้ป่วยที่มีระดับเพอริออสตินในชิ้นเนื้อไตสูง มีค่าการทำงานของไตต่ำกว่าผู้ป่วยที่มีระดับเพอริออสตินต่ำ

ผลการตรวจระดับเพอริออสตินในปัสสาวะ พบว่า ระดับเพอริออสตินในปัสสาวะของกลุ่มผู้ป่วยมีค่าสูงกว่ากลุ่มอาสาสมัครสุขภาพดีอย่างมีนัยสำคัญทางสถิติ ($p<0.05$) และยังพบความสัมพันธ์ระหว่างระดับเพอริออสตินในปัสสาวะกับค่าการทำงานของไตในกลุ่มผู้ป่วย ($p<0.05$) โดยตรวจพบระดับเพอริออสตินในปัสสาวะของผู้ป่วยจำนวน 23 จาก 50 รายและกลุ่มอาสาสมัครสุขภาพดีจำนวน 11 จาก 50 ราย ซึ่งกลุ่มผู้ป่วยที่ตรวจพบระดับเพอริออสตินในปัสสาวะมีค่าการทำงานของไตต่ำกว่ากลุ่มผู้ป่วยที่ตรวจไม่พบระดับเพอริออสตินในปัสสาวะอย่างมีนัยสำคัญทางสถิติ สำหรับผลการตรวจระดับเพอริออสตินในซีรั่ม ไม่พบความแตกต่างระหว่างกลุ่มผู้ป่วย และกลุ่มอาสาสมัครสุขภาพดี นอกจากนี้ ยังไม่พบความสัมพันธ์ระหว่างระดับเพอริออสตินในซีรั่มและระดับเพอริออสตินในปัสสาวะ สำหรับผลการประเมินการตอบสนองต่อการรักษาในกลุ่มผู้ป่วย หลังจากได้รับการรักษาเป็นเวลา 6 เดือน พบว่าไม่มีความแตกต่างของพยาธิสภาพของไต ข้อมูลทั่วไป ค่าการทำงานของไต ยาที่ได้รับ รวมถึงระดับเพอริออสตินในซีรั่มและปัสสาวะ ระหว่างกลุ่มผู้ป่วยที่ตอบสนองและไม่ตอบสนองต่อการรักษา อย่างไรก็ตาม พบระดับเพอริออสตินในปัสสาวะลดลงหลังจากได้รับการรักษาเป็นเวลา 6 เดือน ในกลุ่มผู้ป่วยที่ตอบสนองต่อการรักษา ($p<0.05$)

การวิจัยนี้สรุปได้ว่า เพอริออสตินอาจใช้เป็นตัวบ่งชี้ทางชีวภาพในชิ้นเนื้อไตในกลุ่มผู้ป่วยโรคไตอักเสบลูปัส และผู้ป่วยโรค IgA nephropathy ซึ่งมีความสัมพันธ์กับความเรื้อรังของโรคไต รวมถึงค่าการทำงานของไต และยังสามารถทำนายการดำเนินโรคที่แย่ลงเมื่อเปรียบเทียบกับการย้อมมาตรฐาน โดยเฉพาะในกลุ่มผู้ป่วยที่มีความรุนแรง และความเรื้อรังของโรคต่ำ สำหรับการตรวจระดับเพอริออสตินในปัสสาวะ อาจสามารถทำนายการดำเนินโรคที่แย่ลงในกลุ่มผู้ป่วยโรคไตอักเสบลูปัส และผู้ป่วยโรค IgA nephropathy และยังมีแนวโน้มในการใช้ติดตามผลการตอบสนองต่อการรักษาหลังจากได้รับการรักษาเป็นเวลา 6 เดือน

ภาควิชา	เภสัชกรรมปฏิบัติ	ลายมือชื่อนิสิต .....
สาขาวิชา	การบริบาลทางเภสัชกรรม	ลายมือชื่อ อ.ที่ปรึกษาหลัก .....
ปีการศึกษา	2558	ลายมือชื่อ อ.ที่ปรึกษาร่วม .....

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KEYWORDS: PERIOSTIN / RENAL PATHOLOGY / LUPUS NEPHRITIS / IGA NEPHROPATHY

PEEPATTRA WANTANASIRI: DETECTION OF KIDNEY INJURY BY USING THE CORRELATION OF PERIOSTIN AND RENAL PATHOLOGY IN PATIENTS WITH LUPUS NEPHRITIS AND IgA NEPHROPATHY. ADVISOR: PROF. PORNANONG ARAMWIT, Pharm.D., Ph.D., CO-ADVISOR: MAJ. BANCHA SATIRAPOJ, M.D., 162 pp.

The objective of this study was to examine the correlation between periostin level in kidney tissue, urine and serum samples and renal pathology as well as renal functions in patients with lupus nephritis and IgA nephropathy and to investigate the periostin levels of patients compared with controls. The prediction of clinical response from periostin measurement was also assessed. This study was conducted from April 2013 to February 2015 at the Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand. Fifty patients and 50 healthy controls were included in this study. There were 37 and 13 patients diagnosed with lupus nephritis and IgA nephropathy, respectively. Five normal kidney tissue sections from renal cell carcinoma patients were used as control kidney tissues. The results from periostin immunohistochemistry found that periostin was not detected from control kidney tissue. In contrast, the most common area with positive periostin from patients' kidney tissue was tubular. Periglomeruli, sclerosed glomeruli, interstitial fibrosis and vascular fibrosis were also positive for periostin. The periostin staining was significant correlated with chronicity index and renal functions ($p<0.05$). In addition, worsening renal function was observed in patients with high periostin staining scores compared with low periostin staining scores among patients with low active and low chronic disease.

The results from urine periostin analysis reported a significantly higher level of urine periostin in patients than in healthy controls ($p<0.05$). There was a significant correlation between urine periostin level and renal functions ($p<0.05$). Urine periostin was detected in 23 out of 50 patients and 11 out of 50 healthy controls. Worsening renal function was found in patients with urine periostin detection. In contrast, there was no significant difference in serum periostin level between patients and healthy controls. No correlation was found between serum periostin level and urine periostin level. After 6 months of treatment, there was no statistical difference in baseline renal pathology, characteristic data, renal parameters, treatment, urine periostin level and serum periostin level between patients with response and non-response to therapy. However, there was a significant decrease in urine periostin level after 6 months of treatment in patients with response to therapy ($p<0.05$).

In conclusion, periostin may be a promising tissue biomarker in lupus nephritis and IgA nephropathy patients that is related to chronic kidney disease progression and kidney functions. Periostin staining may be used for predicting worsening kidney disease progression rather than routine staining, especially in patients with low active disease or low chronic disease. Urine periostin measurement may be used for the prognosis of disease progression in lupus nephritis and IgA nephropathy patients. It may be possible to use urine periostin measurement for monitoring response to therapy after 6 months of treatment.

Department:	Pharmacy Practice	Student's Signature	.....
Field of Study:	Pharmaceutical Care	Advisor's Signature	.....
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CONTENTS

Page

Page

Page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

ACEI	=	Angiotensin converting enzyme inhibitors
ACR	=	American College of Rheumatology
ARB	=	Angiotensin receptor blockers
AZA	=	Azathioprine
CI	=	Confidence interval
CKD	=	Chronic kidney disease
CKD-EPI	=	Chronic Kidney Disease Epidemiology Collaboration
CYC	=	Cyclophosphamide
eGFR	=	Estimated glomerular filtration rate
ELISA	=	Enzyme-linked immunosorbent assay
EMT	=	Epithelial-mesenchymal transition
ESRF	=	End-stage renal failure
GC	=	Glucocorticoids
GFR	=	Glomerular filtration rate
H&E	=	Hematoxylin and eosin
IgAN	=	IgA nephropathy
IL	=	Interleukin
ISN/RPS	=	International Society of Nephrology/Renal Pathology Society
IV	=	Intravenous
KDIGO	=	Kidney Disease Improving Global Outcomes
KIM-1	=	Kidney injury molecule-1
L-FABP	=	Liver-type fatty acid binding protein
LN	=	Lupus nephritis
MDCT	=	Mouse distal collecting tubular
MDRD	=	Modification of Diet in Renal Disease

MMF	=	Mycophenolate mofetil
NAG	=	N-acetyl-beta-D-glucosaminidase
NGAL	=	Neutrophil gelatinase-associated lipocalin
NPV	=	Negative predictive value
PBS	=	Phosphate-buffered saline
PCR	=	Polymerase chain reaction
PPV	=	Positive predictive value
ROC	=	Receiver operating characteristics
SLE	=	Systemic lupus erythematosus
SMA	=	Smooth muscle actin
TGF	=	Transforming growth factor

CHAPTER I

INTRODUCTION

1.1 Rationale and Background

Chronic kidney disease (CKD) is a public health problem all over the world. In Thailand, the CKD prevalence is high in Bangkok, the northern and the northeastern regions. Only a few patients are aware that they have kidney disease [1]. The severity of the disease may increase in untreated patients. The greater the severity of the disease, the more risk of death and cardiovascular events there is [2]. Early detection, diagnosis and treatment of underlying causes are important in CKD patients. These may delay the progression of the disease toward end-stage renal disease. Patients with greater end-stage renal disease symptoms are more likely to have lower quality of life [3]. Quality of life is also significantly decreased over time after receiving renal replacement therapy [4].

There are many causes of chronic kidney disease such as diabetes, hypertension, infectious glomerulonephritis, ureteral obstruction, autoimmune diseases and others [5]. The analysis of 3,555 cases of renal biopsy in Thailand found that lupus nephritis is the most prevalent cause of secondary glomerulonephritis (88.5%) [6]. Among patients with asymptomatic urinary abnormalities, IgA nephropathy is the most frequent cause according to histopathological diagnosis in all age groups, especially 15–35 years of age (80%) [7]. Even though the causes of disease are different, there are the common features of chronic kidney disease progression including glomerulosclerosis, tubulointerstitial inflammation, tubulointerstitial fibrosis and tubular

atrophy [8]. These characteristic features lead to kidney function reduction until end-stage renal failure.

Chronic kidney disease development and progression is insidious. Most patients in the early stage are asymptomatic. Abnormal symptoms are present in patients with greater severity. Estimated glomerular filtration rate (eGFR) is the most common measurement for evaluation of renal function and classification of the disease's severity by calculating from serum creatinine. An increase in serum creatinine is one of the most common features in the detection of abnormal renal function. However, there are some limitations. Many factors can affect the level of serum creatinine such as age, sex, race, body habitus, chronic illness and diet [9]. It is not a specific indicator for renal damage and does not represent an abnormality in the pathology of kidney disease [10]. The sensitivity of estimated GFR from Cockcroft and Gault and the Modification of Diet in Renal Disease (MDRD) equation is low for CKD classification [11]. An increase in serum creatinine will be observed when renal function is reduced by more than 50% leading to low sensitivity for diagnosis of early stage CKD. In addition, there are many causes of changes in serum creatinine besides renal function. An increase in serum creatinine or lowering of estimated GFR can occur when there is no change in renal pathology and cannot represent the positioning of abnormal renal pathology [12]. Therefore, the novel biomarker which is more specific to abnormal renal pathology and kidney disease progression should be further investigated.

Another renal function assessment is proteinuria, the most common feature presented in lupus nephritis patients. Proteinuria may be observed before the elevation of serum creatinine and may be used as an early marker for the detection of kidney injury. Abnormality of urinary protein excretion for more than 3 months, with or without a decrease in GFR, is defined as chronic kidney disease [13]. Urine protein excretion can predict a decline in GFR and progression towards end-stage renal failure (ESRF) in non-diabetic proteinuric chronic nephropathies. After 23 months follow-up, overall GFR

decreased by 0.46 ml/min/1.73m^2/month and the progression to ESRF was 17.3%. Higher urinary protein correlated with faster decline in GFR and progression to ESRF [14]. In addition, a correlation between estimated GFR and risk of death and cardiovascular events was also reported after 2.84 years follow-up. The risk of death was increased when eGFR decreased, with the highest hazard ratio of 5.9 in patients with eGFR lower than 15 ml/min/1.73m^2. The results of cardiovascular events were similar to the hazard ratio of 3.4 in patients with eGFR lower than 15 ml/min/1.73m^2 [2]. Early detection of kidney injury will decrease the risk of death and cardiovascular events and also delay the progression towards end-stage renal failure. Since the present kidney function assessment had some limitations with low sensitivity for detecting the abnormality in early-stage kidney disease, it is not specific for renal disease and cannot represent the abnormality of renal pathology. Therefore, searching for new biomarkers should be considered for these reasons.

Periostin is a matricellular protein that was primarily expressed in bone [15]. It is also involved in kidney development [16, 17]. In animal study with 5/6 nephrectomy, periostin mRNA expression was increased after early of kidney injury and still elevated after nephrectomy at 2 days, 2 weeks and 4 weeks. Periostin staining was positive in glomeruli, interstitium and casts and/or sloughed cells in the tubular lumina. Half of the distal tubule was positive for periostin [18]. Urine periostin analysis demonstrated the same results. Urine periostin was undetectable before nephrectomy. After nephrectomy, urine periostin increased over time up to 4 weeks [18]. From this result, urine periostin may be used to distinguish a normal kidney from an injured kidney and may be related to the chronicity of the disease. In a human study, the result of urine periostin analysis was reported similarly. There was a statistically significant difference in urine periostin between healthy controls and chronic kidney disease patients. Urine periostin can be detected in both proteinuric and non-proteinuric patients, with higher levels in non-proteinuric groups, which indicates that periostin may be used as a marker for tubular injury [18]. In addition, no detection of periostin staining was seen in normal

kidneys from immunohistochemistry analysis. In contrast, periostin staining was detected in both animal and human studies with kidney disease [18-20]. Positive periostin was also found in the glomerular tuft and wall of arteries and strongly in interstitial fibrosis in human transplant nephrectomies due to chronic dysfunction [20]. The highest induction was found in proliferative lupus nephritis compared with living donors. Periostin staining was detected in the glomerular tuft and was more diffuse in the interstitial area in lupus nephritis patients with impaired renal function [20]. Periostin was also found to be involved in cell proliferation [20, 21]. Moreover, the role of periostin in fibrosis process was reported. There was a co-expression of periostin and epithelial-mesenchymal transition markers observed in kidney tissue samples from both animal and human with kidney injury [18, 19]. In addition, a reduction in areas with fibrosis and tubular dilation was found in kidney tissue samples from animal with gene deletion of periostin [22]. The results from studies show that periostin may be used as a biomarker for kidney injury that is related to disease progression. It was found in both urine and kidney tissue in patients with chronic kidney disease. In contrast, periostin was not detected in normal kidneys. Due to limitations of conventional assessments of kidney function mentioned before, a specific biomarker, periostin which is related to kidney disease progression should be further investigated.

1.2 Hypotheses

1.2.1 There is a correlation between periostin staining, urine periostin, serum periostin and renal pathology as well as renal functions in patients with lupus nephritis and IgA nephropathy.

1.2.2 Periostin staining, urine periostin and serum periostin are different between patients with lupus nephritis and IgA nephropathy compared with normal controls.

1.2.3 Periostin staining, urine periostin and serum periostin can predict response to therapy after 6 months of treatment.

1.3 Objectives

1.3.1 To examine the correlation of the periostin level in kidney tissue, urine and serum samples and renal pathology as well as renal functions in patients with lupus nephritis and IgA nephropathy.

1.3.2 To examine the level of periostin in kidney tissue, urine and serum samples from patients with lupus nephritis and IgA nephropathy compared with controls.

1.3.3 To predict the clinical response from periostin measurement after 6 months of treatment.

1.4 Scopes

1.4.1 Kidney tissue, urine and blood samples from patients with lupus nephritis and IgA nephropathy were collected during April 2013 to February 2015 at Phramongkutklao Hospital, Bangkok, Thailand. Immunohistochemistry of periostin, periostin mRNA expression, urine periostin and serum periostin were measured to examine the correlation with renal pathology as well as renal functions and compared with normal controls.

1.4.2 After 6 months of treatment, clinical response was assessed and urine samples were collected from patients with lupus nephritis and IgA nephropathy to find out whether periostin can predict clinical response.

1.5 Expected Outcomes

1.5.1 Periostin could be used as a biomarker with strong correlation with renal pathology as well as renal functions in patients with lupus nephritis and IgA nephropathy.

1.5.2 Periostin measurements should distinguish patients with lupus nephritis and IgA nephropathy from normal controls.

1.5.3 Periostin can predict clinical response after 6 months of treatment.

CHAPTER II

LITERATURE REVIEWS

2.1 Lupus Nephritis

Lupus nephritis (LN) is one of the most serious complications in patients with systemic lupus erythematosus (SLE). Approximately 25-50% of patients with SLE have renal involvement presented by urine abnormalities and impairment of renal function. Up to 60% of adults may have renal abnormality later. The clinical features of LN are proteinuria, nephrotic syndrome, granular casts, microscopic hematuria, tubular abnormality and renal function reduction. Proteinuria is the most common feature and tubular abnormality is also present in most patients, usually without symptoms [23]. LN is defined from clinical presentations and laboratory testing that follow American College of Rheumatology (ACR) criteria as a persistent proteinuria greater than 0.5 grams per day or greater than 3+ if quantitation is not performed, or the presence of cellular casts including red cell, hemoglobin, granular, tubular or mixed [24]. According to the review of ACR criteria, a spot urine protein to creatinine ratio of more than 0.5 and active urinary sediment can be substituted. Classification of glomerulonephritis should be made from the histopathology of kidney tissue evaluated by an experienced pathologist. Routine histopathology, immunofluorescence and electron microscopy are recommended for renal biopsy assessment [25].

2.1.1 Renal Biopsy and Histology of Lupus Nephritis

Renal biopsy is an important tool for the evaluation of LN patients. At present, it is common and safely done by nephrologists. It is recommended in patients with renal abnormality defined by increasing serum creatinine without compelling alternative causes or proteinuria more than or equal to 0.5 grams per day plus hematuria or cellular cell cast, or confirmed in patients with proteinuria more than or equal to 1 gram per day [26]. Histological findings from glomeruli, interstitium and renal tubules in kidney tissue are major sources for classification of LN types. These classifications are an initial guide for treatment preparation. The types of LN according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) are classified into six types [27]. Class I (minimal mesangial LN) presents with normal light microscopy but immunofluorescence and electron microscopy finding with immune deposits. Class II (mesangial proliferation LN) presents with mesangial hypercellularity or matrix expansion by light microscopy with immune deposits seen by fluorescence microscopy. Class III (focal LN) is defined by any lesion or scar involving less than 50% of glomeruli. Class IV (diffuse LN) is defined by any lesion or scar involving 50% or more of glomeruli. Subgroup classifications in class III and class IV include active lesions, chronic lesions or both. Class V (membranous LN) presents with subepithelial immune deposits and class VI (advanced sclerosis LN) with 90% or more of globally sclerosed glomeruli without residual activity [27]. In addition, evaluation of activity and chronicity index by a pathologist is also recommended (Table 1). Kidney tissue was assessed for glomerular abnormalities and tubulointerstitial abnormalities. Activity index was assessed from six histologic parameters. Scores were graded as a percentage of the affected area as follows: 0 (absent), 1 (less than 25% of glomeruli affected), 2 (25% to 50% of glomeruli affected), or 3 (more than 50% of glomeruli affected); except for hyaline thrombi or wire loop, glomerular leukocyte infiltration and interstitial inflammation were graded as 0 (absent), 1 (mild), 2 (moderate), or 3 (extensive). Fibrinoid necrosis or karyorrhexis and cellular crescents were given a double weighting score. Chronicity index was assessed from four histologic parameters. Glomerular sclerosis and fibrous crescents were

graded as 0 (absent), 1 (less than 25% of glomeruli affected), 2 (25% to 50% of glomeruli affected), or 3 (more than 50% of glomeruli affected). Interstitial fibrosis and tubular atrophy were graded as 0 (absent), 1 (mild), 2 (moderate), or 3 (extensive). Activity index and chronicity index scores were calculated from the summation of individual scores. The maximum scores for activity and chronicity index are 24 and 12, respectively [28].

Table 1 Activity index and chronicity index

Activity index	Activity score	Chronicity index	Chronicity score
Glomerular abnormalities		**Glomerular abnormalities**	
Glomerular cell proliferation	0-3	Glomerular sclerosis	0-3
Fibrinoid necrosis or karyorrhexis	0-6	Fibrous crescents	0-3
Cellular crescents	0-6		
Hyaline thrombi or wire loops	0-3		
Glomerular leukocyte infiltration	0-3		
Tubulointerstitial abnormality		**Tubulointerstitial abnormalities**	
Interstitial inflammation	0-3	Interstitial fibrosis	0-3
		Tubular atrophy	0-3
Total score	**0-24**		**0-12**

2.1.2 Treatment of Lupus Nephritis

Treatment for class I and class II is not needed for immunosuppressive agents. Patients with class III and class IV require more aggressive glucocorticoids and immunosuppressive agents. Patients with a higher activity score should receive more immunosuppressive agents. In contrast, a higher chronicity score is less likely to respond to immunosuppressive agents [26]. The treatment for class III and IV LN is composed of two phases, including initial and maintenance phases. The aim is to rapidly reduce kidney inflammation by initial intensive treatment, followed by

maintenance treatment. The ACR guideline for LN treatment is shown in Table 2 [26]. Mycophenolate mofetil or intravenous cyclophosphamide along with glucocorticoids is recommended as initiation treatment for class III and IV LN. In patients who fail to respond after initial treatment, rituximab or calcineurin inhibitors may be selected. For maintenance treatment, mycophenolate mofetil or azathioprine is recommended. Patients with class V in combination with class III or class IV should receive the same treatment as class III or class IV. The treatment for class V (pure membranous) and with nephrotic range proteinuria is also shown in Table 2. Patients with advanced sclerosis as in class VI should prepare for renal replacement therapy [26].

Table 2 Treatment for LN class III, IV and V

LN classification	Initiation treatment	Maintenance treatment
Class III and IV	MMF 2-3 g/day for 6 months or	MMF 1-2 g/day ± low dose daily GC or
	CYC 500 mg IV every 2 weeks for 3 months (6 doses) or	AZA 2 mg/kg/day ± low dose daily GC
	CYC 500-1,000 mg/m^2 IV monthly for 6 months (6 doses)	
	Plus	
	GC IV pulse for 3 days, then prednisone 0.5-1 mg/kg/day tapered after a few weeks to lowest effective dose	
Class V	MMF 2-3 g/day for 6 months or	MMF 1-2 g/day or
	CYC 500-1,000 mg/m^2 IV monthly for 6 months (6 doses)	AZA 2 mg/kg/day
	Plus	
	GC pulse followed by prednisone 0.5-1 mg/kg/day	

MMF = mycophenolate mofetil; CYC = cyclophosphamide; GC = glucocorticoids;

IV = intravenous; AZA = azathioprine

2.2 IgA Nephropathy

IgA nephropathy (IgAN) is the most common primary glomerulonephritis in the world. The prevalence varies in different geographical regions. Asians are more prone to IgA nephropathy [29, 30]. The same tendency was also reported in Thailand [7]. Clinical presentations of IgA nephropathy patients are wide-ranging, from isolated hematuria to rapid progressive glomerulonephritis [31]. Most patients are presented with recurrent macroscopic hematuria. Asymptomatic persistent microscopic hematuria was found in about 30-40% of patients. Nephrotic syndrome is uncommon, occurring in only 5% of patients and defined as proteinuria of more than 3.5 g/day combined with edema, hypoalbuminemia and hypercholesterolemia. Less than 5% of patients are presented with acute kidney injury [31].

2.2.1 Renal Biopsy and Histology of IgA Nephropathy

IgA nephropathy is diagnosed by kidney biopsy. Typical features of IgA nephropathy are identified by light microscopy, immunofluorescence and electron microscopy. The most common observations from light microscopy are focal or diffuse expansion of mesangial cells or matrix. Other abnormalities may be seen including diffuse endocapillary proliferation, segmental sclerosis, segmental necrosis and cellular crescent formation [32]. Immunofluorescence demonstrated dominant or co-dominant staining with IgA in mesangial regions of glomeruli with more than trace intensity. IgG and IgM may be present with less intensity than IgA, except for IgM, which may be more intense in sclerotic areas [32]. Electron microscopy identifies with predominantly electron-dense deposits within mesangial regions of glomeruli. Focal or diffuse expansion of mesangial cells, matrix or both may be present. In addition, the change of other features should be identified, including interstitial fibrosis, interstitial inflammation, tubular atrophy, vascular wall thickening, vascular sclerosis or casts within tubules, which may provide prognostic information for patients [33].

Renal biopsy for IgA nephropathy should be reported for four key pathological features known as the Oxford classification: mesangial hypercellularity, segmental glomerulosclerosis, endocapillary hypercellularity and tubular atrophy/interstitial fibrosis. The definitions of each pathological feature in the Oxford classification are described in Table 3 [34]. From the univariate analysis results, segmental glomerulosclerosis, endocapillary hypercellularity and tubular atrophy/interstitial fibrosis strongly impacted on doubling creatinine and end-stage renal disease. These features were also associated with a higher amount of proteinuria and lower eGFR, suggesting that the Oxford classification may be useful for renal prognosis in IgA nephropathy patients [35]. However, more validations should be performed.

Table 3 Definition of each pathological feature in Oxford classification

Pathological features	Definition	Score
Mesangial hypercellularity	< 4 Mesangial cells/mesangial area=0	M0 ≤ 0.5
	4-5 Mesangial cells/mesangial area=1	
	6-7 Mesangial cells/mesangial area=2	M1 > 0.5 [a]
	> 8 Mesangial cells/mesangial area=3	
Segmental glomerulosclerosis	Any amount of the tuft involved in sclerosis, but not involving the whole tuft or the presence of an adhesion	S0-absent S1-present
Endocapillary hypercellularity	Hypercellularity due to increased number of cells within glomerular capillary lumina causing narrowing of the lumina	E0-absent E1-present
Tubular atrophy/ interstitial fibrosis	Percentage of cortical area involved by the TA or IF, whichever is greater	T0 = 0-25% T1= 26-50% T2 > 50%

[a] Mesangial score should be assessed in periodic acid-Schiff-stained sections. If more than half the glomeruli have more than three cells in a mesangial area, this is categorized as M1. Therefore, a formal mesangial cell count is not always necessary to derive the mesangial score.

2.2.2 Treatment of IgA Nephropathy

According to the 2012 Kidney Disease: Improving Global Outcomes (KDIGO) guidelines, treatment recommendations are focused on primary IgA nephropathy. The control of blood pressure and proteinuria was recommended for better kidney survival. For anti-proteinuric therapy, angiotensin converting enzyme inhibitors (ACEI) or angiotensin receptor blockers (ARB) are recommended for patients with proteinuria greater than 1 g/day and also suggested for patients with proteinuria between 0.5-1 g/day. The dose of treatment can be titrated as far as tolerated until proteinuria is less than 1 g/day [36]. The target blood pressure is less than 130/80 mmHg in patients with urinary protein excretion of less than 1 g/day but less than 125/75 mmHg when initial urinary protein excretion is more than 1 g/day. In the case of persistent urinary protein excretion of more than 1 g/day after 3 to 6 months of proper supportive treatment including ACEI or ARB treatment and blood pressure control, 6 months of corticosteroid therapy may be suggested for patients with eGFR of more than 50 ml/min/1.73m^2. Treatment with fish oil is also suggested for patients with persistent proteinuria. There is no suggestion for using the following treatments in IgA nephropathy: corticosteroids together with cyclophosphamide or azathioprine (except for crescentic IgA nephropathy with rapid deterioration of kidney function), immunosuppressive therapy in patients with eGFR of less than 30 ml/min/1.73m^2 (except for crescentic IgA nephropathy with rapid deterioration of kidney function), mycophenolate mofetil, antiplatelet agents and tonsillectomy [36].

2.3 Assessment of Kidney Function

2.3.1 Creatinine

Creatine synthesis occurs primarily in the liver and is released into the circulation followed by being actively transported into the muscle, which contains approximately 98% of the total body creatine pool. Within muscle, the creatine pool is turned to creatinine daily [37]. The size of the creatine pool is influenced by dietary sources such as meat. Ingestion of meat increases creatinine generation. Moreover, cooking causes a significant increase in creatinine production [38]. Other factors that affect the total muscle mass and creatinine generation are age, sex, race, body habitus and pathophysiologic stages, which are associated with muscle mass reduction. People with low muscle mass such as women, children, the elderly, malnourished patients and cancer patients are associated with declining creatinine production [9, 39].

An ideal filtration marker is a substance that is freely excreted by glomerular filtration without tubular reabsorption or secretion. The clearance of an ideal filtration marker can provide an accurate estimation of glomerular filtration rate [39]. Creatinine is not protein bound and is freely filtered through glomeruli. It is not metabolized by the kidneys and is physiologically inert. These properties show that creatinine may be suitable for use as an ideal filtration marker except for tubular secretion and reabsorption. In normal individuals, there is a tubular secretion of creatinine of 10-40% of excreted creatinine. Moreover, the increase in tubular secretion is found in renal disease patients to be as high as 50-60%. Tubular reabsorption is also observed when the urine flow rate is very low as a result of passive diffusion from lumen to blood leading to lower creatinine clearance and higher serum creatinine [40].

Glomerular filtration rate (GFR) is the rate at which ultrafiltration of glomerular capillary blood passes through the capillary wall of Bowman's capsule due to pressure

[40]. Direct measurement of GFR in humans is not possible. The calculation of GFR from renal clearance of an ideal filtration marker has been considered. Inulin is an ideal filtration biomarker that is freely filtrated in glomeruli, not reabsorbed and secreted, nor metabolized by kidney and physiologically inert. Nevertheless, it is not practical to use this measurement in clinical practice because it is time-consuming, costly and cumbersome [40]. Creatinine is another marker that meets some ideal filtration biomarker criteria. Estimation of GFR from creatinine clearance is more practical. The advantage of estimated GFR from creatinine is that there is no need for any injection of substances. A 24-hour urine collection is used instead. However, the main problem with this method is that it is incomplete and errors from sample collection lead to the underestimation of renal functions. Overestimation of urine creatinine concentration is observed from tubular secretion of creatinine even in normal renal function [41]. Most chronic kidney disease patients also report overestimation of GFR when using creatinine clearance for GFR determination compared with clearance of inulin (true GFR) caused by tubular secretion of creatinine [42]. A higher amount of tubular secretion of creatinine is detected in patients with moderate GFR reduction. In a longitudinal study, patients with deterioration of kidney disease reported a 33% reduction of creatinine clearance and 29% reduction of reciprocal of serum creatinine. However, the true GFR from inulin clearance represents a 48% reduction of renal function with an increase in serum creatinine from 1.4 to 2.3 mg/dl. In contrast to remission patients, there are 13% and 12% increases in creatinine clearance and reciprocal of serum creatinine, respectively. The true GFR represents a 33% increase in inulin clearance with a serum creatinine reduction from 1.6 to 1.4 mg/dl [42]. According to the results, a reduction in GFR by more than half may occur before an increase in serum creatinine. Estimation of GFR from serum creatinine cannot represent kidney injury until greater impairment of glomerular function has occurred. From individual clearances of inulin and creatinine comparison, most patients with a modest decrease of true GFR to 40 ml/min/1.73 m^2 still had a creatinine clearance within a normal range, which provided the same results of

serum creatinine concentration that were also within a normal range [42]. This finding suggested the insensitivity of serum creatinine for estimating GFR.

Many equations were developed for the estimation of creatinine clearance from serum creatinine concentration to reduce the disadvantages and inconvenience from urine collection including Cockcroft and Gault, Modification of Diet in Renal Disease (MDRD) and the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equations. The Cockcroft and Gault equation was developed from 249 patients without renal or liver disease. The correlation coefficient between predicted and mean measured creatinine clearance was 0.83 [43]. The bias from using Cockcroft and Gault for estimating GFR ranged from -14% to 25% [13]. The MDRD equation was created from diverse causes of chronic kidney disease populations by using regression analysis including serum creatinine and factors that affected creatinine excretion such as age, sex and ethnicity [44]. The results from the MDRD equation to estimate GFR in chronic kidney disease and healthy controls reported greater accuracy of the MDRD equation for estimating GFR in chronic kidney disease patients than in healthy controls. Underestimated GFR was found in 29% of healthy controls and 6.2% of CKD patients. The same results were also found by using the Cockcroft and Gault equation with underestimated GFR in 27% of healthy controls and 5.9% in CKD patients [45]. The limitations of the MDRD equation were that there was not validated for some subgroups such as persons without renal disease, persons with type 1 diabetes and persons with type 2 diabetes who receive insulin, children, the elderly, pregnant women, patients with serious comorbid conditions and renal transplant recipients. It is not accurate in patients whose creatinine is not in a steady state [44]. The classification of CKD by using estimated GFR from the Cockcroft and Gault and MDRD equations was also evaluated. The true classification of CKD stage was found in 61.6% and 57.1% of patients when calculated by the Cockcroft and Gault and MDRD equation, respectively. The highest percentage of patients who were classified in the right category was reported in CKD stage 3 from both equations [11]. A tendency of both formulas was underestimation at

high measured GFR and overestimation at low measured GFR. The classification of CKD from both equations provided low sensitivity (<73%) in all GFR groups, high specificity (>92%) in CKD stages 1, 4 and 5, low positive predictive value in all stages except for stage 1 and good negative predictive value (>91%) in patients with GFR of less than 60 ml/min/1.73 m^2 [11]. Another equation that developed from several studies is the CKD-EPI equation. The accuracy for estimating GFR of less than 60 ml/min/1.73 m^2 was equal to the MDRD equation. However, greater accuracy of the CKD-EPI equation was found in subgroup analysis with estimated GFR of more than 60 ml/min/1.73 m^2 [46]. True classification by using the CKD-EPI equation was reported more correctly than with the MDRD equation (63% vs 34%). Moreover, the CKD-EPI equation reported less bias and greater precision and accuracy than the MDRD equation [46].

2.3.2 Proteinuria

In patients with renal disease, kidney function assessment from urinary protein excretion should be evaluated. Proteinuria may be observed before the elevation of serum creatinine, which may be used as an early marker of kidney injury in glomerular diseases. Abnormality of urinary protein excretion for more than 3 months, with or without a decrease in GFR, is defined as chronic kidney disease [13]. Normal urinary protein excretion is between 30 and 150 mg/day. Approximately 30 mg of excreted protein are albumin. Most of the albumin that enters through glomeruli is reabsorbed in the proximal tubule. The detection of proteinuria represents the abnormality of the charge and size selectivity barrier at the glomerular basement membrane [41]. Proteinuria is defined as a total protein excretion of more than 300 mg/24 hour (referred to as albuminuria if albumin is the only protein measured) [47]. A urine dipstick test is the semi-quantitative method used to identify proteinuria. In patients with a positive dipstick test, 24-hour urine collection should be considered for further measurement. This averages the variation in protein excretion throughout a day. However, this method is cumbersome and error from over or under urine collection may occur. [41]. Spot urine samples for measurement of the albumin or protein to creatinine ratio are used instead

with more convenience and more accuracy from the protein excretion normalization with glomerular filtration [47]. Urine collection on the first morning is recommended because this is correlated with 24-hour protein excretion. The normal ratio is less than 30 mg of albumin or less than 200 mg of protein per gram of urine creatinine [41].

2.4 Biomarkers Related with Tubular Damage in Chronic Kidney Disease

2.4.1 Neutrophil Gelatinase-Associated Lipocalin (NGAL)

NGAL is a small protein with a molecular mass of approximately 25 kD. In normal condition, there is a proximal tubular reabsorption of NGAL during glomerular filtration. Low levels of plasma or urinary NGAL can be detected in this condition. During acute kidney injury, there was an impairment of proximal tubular reabsorption from tubular damage leading to a higher level of NGAL detection [48]. In chronic kidney disease, there was a significantly higher level of NGAL in both serum and urine compared with healthy controls. The correlation between eGFR and serum NGAL as well as urinary NGAL was also observed in both univariate and multivariate analysis [49]. This biomarker was also associated with progression of kidney disease. During follow-up, patients with renal disease progression reported higher level of serum NGAL and urinary NGAL at baseline than those without [49, 50]. Faster progression to endpoint was observed in patients with a high level of both serum NGAL and urinary NGAL. In addition, NGAL in both samples was also an independent predictor of chronic kidney disease progression [49]. A greater renal survival rate was also found in patients with low serum NGAL than in those with high serum NGAL [50].

In type 1 diabetic patients, a significantly higher level of urinary NGAL was found than in controls. Subgroup analysis demonstrated a significantly higher level of urinary NGAL only in microalbuminuric patients than in controls. No significant difference was found in normoalbuminuric patients. However, when comparing normoalbuminuric and

microalbuminuric patients, there was a significantly higher level of urinary NGAL in microalbuminuric patients than in normoalbuminuric patients [51]. The same results were reported in type 2 diabetic patients with different degrees of albuminuria. There was a significant increase of NGAL in both serum and urine samples from patients with normoalbuminuria and microalbuminuria compared with controls. Only urine NGAL was significantly higher in microalbuminuric than in normoalbuminuric patients. The highest serum and urinary NGAL levels were also observed in diabetic nephropathy patients. In addition, a significant correlation of NGAL level from serum and urine was found. A correlation between serum NGAL as well as urinary NGAL and renal functions including serum creatinine and eGFR was also reported [52].

In an animal study with antibody-induced nephritis, there was an up-regulation of NGAL mRNA expression in kidney tissues with statistical difference at day 14 compared with controls. The same results were observed from the immunohistochemistry analysis of NGAL within kidney tissue, especially in tubular epithelial cells. In addition, a strong significant correlation between NGAL mRNA expression and histopathological score was reported. A tight significant correlation between urinary NGAL and kidney NGAL was also observed, suggesting the source of urine NGAL secretion from kidney tissue. The main histological feature related to urinary NGAL was found in tubules. Moreover, animals without NGAL gene represented the improvement of renal histological features together with lower proteinuria. This result demonstrated that the presence of NGAL leads to the worsening of kidney structure damage [53].

In systemic lupus erythematosus patients, there was also a significantly higher level of urinary NGAL in patients with renal involvement or active lupus nephritis than in patients without renal involvement. Urinary NGAL was significantly correlated with serum creatinine and creatinine clearance. In contrast, no correlation was observed between urinary NGAL and proteinuria or serum albumin. Urinary NGAL was also a significant predictor of renal disease activity and renal flares in patients with biopsy-proven

nephritis. In addition, it was also a predictor in systemic lupus erythematosus patients with renal involvement [54, 55]. In biopsy-proven lupus nephritis patients, there was a significantly higher level of urinary NGAL in patients with active lupus nephritis than in those with inactive lupus nephritis. Urinary NGAL was also correlated with the duration of lupus nephritis. From multiple logistic regression analysis, serum creatinine and renal disease activity were independent predictors of urinary NGAL level [56]. In addition, a significantly higher of urinary NGAL was observed in patients with renal flares than in those without. Urinary NGAL was found to be a predictor of renal flares [57]. According to receiver operating characteristic curve analysis, urinary NGAL was a better biomarker than anti-dsDNA antibody titer for identifying systemic lupus erythematosus patients with renal involvement or with active lupus nephritis [55, 56]. Moreover, high sensitivity and specificity of urinary NGAL for predicting renal flares was also reported [57].

In IgA nephropathy patients, slight NGAL staining was found in proximal tubule from normal controls and patients with low renal disease severity. In contrast, strong positive NGAL staining was detected in proximal tubules from IgA nephropathy patients with greater disease severity. Positive NGAL was not observed in glomeruli and interstitial cells, suggesting a specific induction of NGAL in proximal tubules [58]. However, the presence of pathological abnormalities including glomerulosclerosis, the severity of interstitial fibrosis/tubular atrophy and mesangial hypercellularity were not different between patients with and without NGAL staining. In a comparison of renal function, the development to end-stage renal disease and renal survival were also not statistically different between patients with and without NGAL staining. In addition, the proportion of patients with NGAL staining was not significantly different for predicting the progression of kidney disease [59]. According to urine NGAL analysis, there was an increment of urinary NGAL in patients compared to healthy controls with the most prominent increase in patients with greater disease severity. Urinary NGAL was also correlated with some pathological features including glomerular mesangial proliferation and tubulointerstitial injury. A significant positive correlation between the intensity of

tubular epithelial cell staining and urinary NGAL was also observed in patients with greater disease severity [58]. No significant association between urinary NGAL level and degree of interstitial fibrosis/tubular atrophy was found [60]. However, urinary NGAL was an independent predictor of tubulointerstitial injury in IgA nephropathy patients with more severity of disease [58]. In addition, a correlation was found between urinary NGAL and renal function including urinary protein output, serum albumin and eGFR, suggesting the potential of urinary NGAL as a biomarker that is involved in glomerular filtration function and histopathological changes in IgA nephropathy patients [58, 60]. In patients who responded to treatment, a significant reduction of urinary NGAL was also observed. In contrast, a high level of urinary NGAL was found in patients who did not respond to treatment. These results suggested that urinary NGAL may be used as an indicator of response to treatment [58]. According to serum NGAL analysis, there was no statistically significant difference between IgA nephropathy patients and controls [58]. However, a correlation between serum NGAL and renal functions such as creatinine, eGFR and urine protein to creatinine ratio was observed [60, 61]. There was a statistically significant higher level of creatinine and urine protein to creatinine ratio and lower level of eGFR and serum albumin in patients with a high plasma NGAL level. [61]. A significant reduction of renal survival was also reported in IgA nephropathy patients with high serum and urinary NGAL levels [60].

2.4.2 Kidney Injury Molecule-1 (KIM-1)

KIM-1 is a transmembrane tubular protein that cannot found in normal kidney. In contrast, it is detectable in kidney injury in both tissue and urine samples. In chronic kidney disease patients, most of KIM-1 positive tubules were also positive for proximal tubular markers, suggesting the main localization of KIM-1 in proximal tubules. Supporting this result, co-localization with a distal tubular marker was not observed in kidney tissue from these patients. In addition, the expression of KIM-1 was correlated with fibrosis and inflammation in both glomerular and interstitial areas [62]. Gene expression of KIM-1 within kidney tissue was also significantly correlated with

tubulointerstitial fibrosis and tubular damage [63]. According to urine KIM-1 analysis, there was a significantly higher urine KIM-1 level in patients than in controls. A significant correlation was found between urinary and kidney tissue KIM-1 expression in these patients. There was also a significant association between urine KIM-1 and inflammation of kidney tissue in both glomerular and interstitial areas. In addition, a correlation between KIM-1 expression and kidney functions including creatinine clearance and eGFR was found in both urine and tissue samples [62].

In both type 1 and type 2 diabetic patients, there was an increase in urine KIM-1 in patients with a higher degree of proteinuria. There was a significantly higher level of urine KIM-1 in microalbuminuria patients than in normoalbuminuria patients and controls. The highest urine KIM-1 level was found in patients with macroalbuminuria, with a significant difference from microalbuminuria and normoalbuminuria patients and controls [64-66]. Urinary KIM-1 level was also correlated with urine albumin excretion, duration of diabetes and hemoglobin A1C level [65, 66]. Moreover, the prediction of kidney disease progression from urinary KIM-1 was also evaluated. After 2-year follow-up of microalbuminuria patients, there was a significantly lower baseline urinary KIM-1 level in patients with regression of kidney disease [64]. In addition, urinary KIM-1 was also a predictor of declining eGFR after 5 years of follow-up. Patients with a higher level of urinary KIM-1 reported a greater eGFR reduction. The factors that affected the progression to macroalbuminuria from microalbuminuria patients were also assessed. However, urinary KIM-1 level did not predict these results [65].

In active lupus nephritis patients, KIM-1 was detected in the dilated tubules near the fibrosis area within kidney tissues. In contrast, no detection was found in inactive lupus nephritis patients. The number of tissue KIM-1 positive cells was also correlated with mesangial proliferation, glomerular fibrosis and interstitial inflammation. A significantly higher level of KIM-1 in both urine and kidney tissue samples from active lupus nephritis patients was found than in inactive lupus nephritis patients. In addition,

there was a more significant increase of urinary KIM-1 in active lupus nephritis patients than in healthy controls. A positive correlation between urinary KIM-1 and proteinuria as well as tubular damage was also observed. Moreover, urinary KIM-1 at baseline was also correlated with renal functions including eGFR and serum creatinine after 6 to 8 months of treatment [67].

In IgA nephropathy patients, KIM-1 expression in kidney tissues was also detected in the dilated tubules near the fibrosis area. Tubular KIM-1 expression was significantly positive correlated with urinary KIM-1 [68]. There was also a significantly higher level of urinary KIM-1 in IgA nephropathy patients than in healthy controls [68-70]. A higher level of urinary KIM-1 was found in patients with greater severity of mesangial proliferation, tubular atrophy, interstitial fibrosis and interstitial infiltration. In addition, the presence of some pathological abnormalities including crescents or endocapillary proliferation was also related to a higher level of urinary KIM-1. According to subgroup analysis between patients with high and low urine KIM-1 levels, the proportion of patients with endocapillary proliferation, global sclerosis and crescents was significantly higher in patients with a high urine KIM-1 level. Greater severity of some pathological features including mesangial proliferation, tubular atrophy, interstitial fibrosis and interstitial infiltration together with the declining of renal functions were observed in patients with a high urine KIM-1 level [68]. In addition, there was a proportional increase in urinary KIM-1 in patients with greater histopathological severity and tubulointerstitial inflammation [70]. A correlation between urinary KIM-1 and renal parameters including serum creatinine, proteinuria and creatinine clearance was also observed [68, 69]. Urine KIM-1 excretion was also an independent predictor of end-stage renal disease [69]. The possibility of KIM-1 being a biomarker for prediction of response to therapy was reported. A significant reduction of urinary KIM-1 level was observed after 24 months of treatment. Subgroup analysis according to baseline KIM-1 level reported the same results in patients with moderate and high baseline urine KIM-1 levels [71].

2.4.3 Liver-type fatty acid binding protein (L-FABP)

L-FABP is a free fatty acid binding protein that is expressed in proximal tubules. In humans with different types of renal disease, a significant correlation between urinary L-FABP and tubulointerstitial damage within kidney tissue was also observed. Greater of urinary L-FABP was reported in patients with more severe tubulointerstitial damage [72]. There was a statistically higher level of urinary L-FABP in patients with different types of chronic kidney disease than in healthy controls. An increment of urinary L-FABP was also observed in patients with greater proteinuria. A correlation between urinary L-FABP and renal functions including serum creatinine, creatinine clearance and urinary protein was reported [72-74]. In addition, urine L-FABP excretion was related to the progression of kidney disease. After 5 years of follow-up, patients were classified as progression and non-progression of renal disease according to the declining eGFR. The level of baseline urinary L-FABP was higher in patients with progression of kidney disease compared with non-progression group. Urinary L-FABP was also correlated with progressive renal function reduction together with serum creatinine, uric acid, urine protein excretion and eGFR. According to logistic regression analysis, higher levels of baseline urine L-FABP and serum creatinine were risk factors for disease progression [74]. In addition, a significantly higher level of urinary L-FABP was detected in chronic kidney disease patients with progression to end-stage renal disease or cardiovascular events [75]. The same results were reported in IgA nephropathy patients. There was a statistically significant higher level of urinary L-FABP in patients than in healthy controls [76, 77]. After 24-month follow-up in non-proteinuria patients, a significant increase in urinary L-FABP was observed in patients with proteinuria [76]. Moreover, there was a significant reduction of urinary L-FABP after 3 months of treatment with the angiotensin receptor blocker and the angiotensin-converting enzyme inhibitor or combination treatment [77].

In a diabetic animal model, there was a significantly higher level of human L-FABP gene and protein expression in kidney tissue from diabetic transgenic mice than in control transgenic mice at 8 weeks. Immunohistochemistry of human L-FABP found that the cytoplasm of the proximal tubules showed positive staining for human L-FABP in transgenic mice. In addition, urinary human L-FABP level was significantly higher in diabetic transgenic mice than in control transgenic mice at 8 and 14 weeks [78]. In diabetic patients, there was a significant increase in urinary L-FABP in patients with macroalbuminuria compared with normoalbuminuria and microalbuminuria. A significant correlation between urinary L-FABP and urinary albumin as well as eGFR was reported in both univariate and multivariate analysis [79]. An increase in urinary L-FABP was observed in patients with progression of diabetic nephropathy after 4 years of follow-up. Greater urinary L-FABP was found in patients with a higher degree of proteinuria with statistically significant difference than in healthy controls. The highest urinary L-FABP level was also reported in patients with end-stage renal failure [80]. In addition, there was a significant increase in urinary L-FABP in normoalbuminuria patients who developed microalbuminuria and macroalbuminuria after 18 years of follow-up compared with persistent normolbuminuric patients. Urinary L-FABP also predicted the development of microalbuminuria and mortality in diabetic patients [81]. A high level of urinary L-FABP was also a predictor of progression of diabetic nephropathy [80]. In addition, urinary L-FABP was an independent predictor after subgroup analysis in normoalbuminuria, microalbuminuria and macroalbuminuria patients with progression to microalbuminuria, macroalbuminuria and end-stage renal failure [82].

2.4.4 N-acetyl-beta-D-glucosaminidase (NAG)

NAG is a lysosomal enzyme, mostly found in the proximal renal tubular cells. In patients with different types of glomerulonephritis, there was a significantly higher level of urinary NAG than in healthy controls. A significant correlation was found between urinary NAG and proteinuria in patients with minimal change disease, diffuse

proliferative glomerulonephritis, mesangiocapillary glomerulonephritis, membranous nephropathy, IgA nephropathy and lupus nephritis. In contrast, urinary NAG was not correlated with serum creatinine in any type of kidney disease [83, 84]. In children with chronic kidney disease, there was a significant negative correlation between urine NAG secretion and creatinine clearance as well as eGFR. In addition, subgroup analysis according to chronic kidney disease stage found that urinary NAG was significantly correlated with creatinine clearance in stage 1 to stage 3 [85].

In type 1 diabetic patients, there was a significantly higher level of urinary NAG in normoalbuminuria patients than in controls as well as in microalbuminuria patients compared with normoalbuminuria patients. After 2-year follow-up, patients with microalbuminuria were classified into 3 groups according to albumin excretion rate as regression, stable and progression of disease. Urinary NAG in patients with regression was significantly lower than in patients with stable disease. A greater tendency for urinary NAG to increase in patients with disease progression than in patients with regression was observed. In addition, the highest percentage of patients with regression was found among those in the lowest quartile of baseline urinary NAG level. A reduction in the percentage of patients with regression was observed in patients with increased in baseline urine NAG excretion [64]. The association between urinary NAG and albuminuria was reported not only in type 1 diabetic patients but also in type 2 diabetic patients. In type 2 diabetic patients with microalbuminuria, the level of urine NAG excretion was significantly higher than in normoalbuminuria patients or healthy controls [84, 86]. The proportional increase in urinary NAG was also observed with a greater degree of proteinuria. There was a significantly higher level of urine NAG excretion in macroalbuminuria than in microalbuminuria patients. The same result was found in microalbuminuria compared with normoalbuminuria patients. A significantly higher level of urinary NAG in all degrees of proteinuria was reported compared with healthy controls. Urinary NAG was also significantly positively correlated with albumin to creatinine ratio, serum creatinine, hemoglobin A1C and disease duration. A negative

correlation with eGFR was found in subgroup analysis of macroalbuminuria patients [87-89]. In addition, there was a significantly higher level of urine NAG excretion in diabetic patients with poor metabolic control than in those with good metabolic control [90].

In children with systemic lupus erythematosus, subgroup urinary NAG level analysis between patients with biopsy-proven lupus nephritis and those without nephritis was performed. There was a significantly higher level of urinary NAG in lupus nephritis patients than in those without [91]. In adult systemic lupus erythematosus patients with proteinuria, urine NAG excretion was also significantly higher than in healthy controls [92, 93]. A tendency for higher urinary NAG was found in patients with renal involvement. In addition, urinary NAG was also a predictor of the severity of renal involvement [93]. Subgroup analysis according to urinary protein level found that urinary NAG was higher in patients with nephrotic-range proteinuria. There was also a strong correlation between urinary NAG and proteinuria. In addition, subgroup urinary NAG analysis according to histopathological severity was also evaluated. However, a significant difference in urinary NAG level was not observed [92]. In addition, there was a study that investigated the change in urinary NAG level after treatment. In lupus nephritis patients, the baseline urinary NAG level was significantly higher than in controls. After 30 days of treatment, there was a significant reduction of urinary NAG level and proteinuria together with an increase in eGFR compared with 7 days of treatment [94].

2.5 Periostin and Kidney Injury

Periostin, osteoblast specific factor 2, is a 90 kDa secreted protein that was first cloned from mouse osteoblastic cell line. It was primarily expressed in bone and weakly expressed in the lung. No periostin has been found in other tissues including kidney [15]. Periostin is also involved in kidney development. It can be found in nephrogenic zones of one-day-old rats. In addition, temporary periostin expression was also reported during nephrogenesis from one-day-old to 10-day-old rats [16]. Periostin expression was also found in embryos. At embryonic day 13.5, the periostin was highly expressed in the outer surrounding kidney. The strong expression of periostin was also observed in the mesenchyme surrounding the ureter at embryonic day 15.5 and 17.5. Exogenous periostin inhibited ureteral branches and the glomerular number. These results suggested that periostin may be involved in branching morphogenesis and nephrogenesis [17].

2.5.1 Immunohistochemistry of Periostin in Kidney Tissue

Immunohistochemistry is a special technique generally used to identify the positioning of the marker of interest in several tissues. The results from the immunohistochemistry of periostin in kidney tissues found that there was no detection of periostin in normal kidney tissue [18, 22, 95]. In contrast, periostin staining was observed in both animal and human injured kidneys. In animals with some part of the kidney removed, periostin staining was found in the cytoplasm, especially in tubular epithelial cells. Periostin staining was also found in fragments of tubular cell in the tubular lumen, interstitial area and glomeruli with abnormal function [18]. Media of renal vessels were also positive for periostin found in animals with hypertensive nephropathy [19]. Greater intensity and spreading of periostin were reported in kidney tissue from chronic injured kidneys including streptozotocin-induced diabetic nephropathy, ureteral

obstruction and a hypertensive nephropathy animal model [18, 19, 22]. In addition, periostin staining was also observed from aging mice with diabetes nephropathy [18].

In human studies, periostin staining was detected in glomerular, interstitium, vascular and tubular areas in injured kidney tissue. In patients with advanced diabetic nephropathy, positive periostin was observed in nodular glomerulosclerosis, periglomerular fibrosis, interstitial fibrosis areas and both atrophic and non-atrophic tubular epithelial cells [96]. The same result of a periostin positive area was also reported from chronic allograft nephropathy patients, especially in areas with fibrosis in both glomerular and interstitial areas [19, 95]. In patients with autosomal dominant polycystic kidney disease, periostin was detected in the cyst epithelial cells and extracellular matrix in the basal surface of the cyst [21]. In patients with different proteinuric glomerulopathies, the quantitative periostin positive area was assessed in both glomerular and interstitial areas. There was a significantly higher percentage of positive periostin areas in both glomerular and tubulointerstitial areas from patients with eGFR lower than 30 ml/min compared with eGFR of more than 60 ml/min [20]. More intensity and diffusion of periostin staining, particularly in interstitial fibrosis areas, was noted in patients with impaired renal function. Moreover, there was a statistically significant negative correlation between periostin positive areas and eGFR in both glomerular ($r = -0.472$, $p=0.01$) and tubulointerstitial areas within kidney tissue ($r = -0.695$, $p < 0.001$) [20].

2.5.2 Periostin mRNA Expression in Kidney Injury

Periostin mRNA expression was detected in kidney tissues from many types of kidney injury such as nephrectomy, diabetic nephropathy and hypertensive nephropathy from animal models [18, 19]. Periostin mRNA expression from injured kidney tissue was statistically higher than in control kidney tissue. Moreover, the increase in periostin mRNA expression increased further over time after chronic kidney injury [18, 19, 22]. In animals that underwent nephrectomy, periostin mRNA expression

statistically increased at 2 days, 2 weeks and 4 weeks after the nephrectomy compared with normal kidneys (3.84-fold, 9.57-fold and 11.05-fold, respectively). The results from periostin protein analysis were similar [18]. In rats with progressive hypertensive renal disease, periostin mRNA expression was elevated 13-fold after 6 weeks and 18- fold after 10 weeks with a significant difference from control kidney tissue. Moreover, after 4 weeks of treatment, the expression of periostin was still statistically higher in animals with deterioration of kidney disease than in the remission group [19].

In human studies, the induction of periostin mRNA expression in glomeruli was also reported in patients with progressive glomerulopathies including lupus nephritis, focal segmental glomerulosclerosis, membranous nephropathy, IgA nephropathy and minimal change disease. However, a statistically significant difference was observed in patients with lupus nephritis and focal segmental glomerulosclerosis compared with living donors [20]. Periostin mRNA expression was also evaluated in tubulointerstitium kidney tissues from patients with focal segmental glomerulosclerosis and membranous nephropathy with different renal function. The results found that the highest periostin mRNA expression was found in patients with eGFR lower than 30 ml/min with statistical difference from patients with eGFR of more than 60 ml/min [20]. In addition, there was a statistically significant negative correlation between periostin mRNA expression in both glomerular and tubulointerstitial areas and eGFR from patients with several types of glomerulonephropathies [20]. A correlation between periostin mRNA expression and other variables including markers of kidney disorder was also reported from both animal and human studies and is summarized in Table 4 [19, 20].

Table 4 The correlation between periostin mRNA expression and other variables

Variables	Correlation between periostin mRNA expression and other variables
Animals with hypertensive nephropathies	
• Plasma creatinine	0.68**
• Proteinuria	0.71**
• Renal blood flow	-0.64**
Patients with glomerulonephropathies	
• Estimated glomerular filtration rate	Glomeruli : -0.18*
	Tubulointerstitial : - 0.47**
• Proteinuria	NS
• Age	NS
Patients with focal segmental glomerulosclerosis and membranous glomerulopathy	
• Estimated glomerular filtration rate	Tubulointerstitial: -0.374*

*p-value < 0.05; ** p-value < 0.001; NS = no significant

2.5.3 Periostin and Renal Fibrosis

Tubulointerstitial injury and fibrosis are the common characteristics leading to end-stage renal failure [97]. There are many features of tubulointerstitial damage including infiltration of inflammatory cells, tubular atrophy and interstitial fibrosis. Epithelial-mesenchymal transition (EMT) is considered as the most important process involved in the progression of kidney diseases [8]. It is a stepwise process initiated by loss of tubular epithelial cell-cell adhesion properties followed by transition of epithelial cells to myofibroblasts. Disappearance of epithelial markers along with *de novo* expression of mesenchymal markers such as α-smooth muscle actin (α-SMA) was observed. The disruption of the tubular basement membrane by matrix metalloproteinase enzyme allowed myofibroblasts to migrate and invade the interstitial area [98]. The imbalance between the production of extracellular matrix protein and its

degradation leads to the accumulation of extracellular matrix protein, and eventually the deterioration of renal function [99, 100].

Tubular epithelial cells and interstitial myofibroblasts are the main cell types of EMT divided by the tubular basement membrane. These cells present with different morphology, markers and locations in kidneys. In normal kidneys, tubular epithelial cells are attached to each other by intercellular adhesion molecules such as E-cadherin which maintains the structure of renal epithelium and controls cell polarity. In chronic kidney disease, tubular epithelial cells are activated by growth factors or proteases that are released from mononuclear cells or interstitial fibroblasts [101]. The principal inducer that drives this process is transforming growth factor-β (TGF-β). In a normal rat kidney tubular epithelial cell line, the epithelial cobblestone morphology was observed in normal condition. In contrast to the presence of TGF-β, the morphology of cells was totally changed including hypertrophy, a lack of epithelial polarity with elongated shape, disconnection with other cells and more invasiveness. The increase in cell number under morphological change was reported when the dosing of TGF-β was increased. These transformations of morphology were also inhibited after adding a neutralizing anti-TGF-β antibody [102]. Moreover, TGF-β induced both α-SMA mRNA expression and the percentage of α-SMA positive cells as a dose-dependent tendency. The immunohistochemistry of α-SMA showed a strong α-SMA staining in cells with hypertrophy, an elongated shape and an invasive pattern. The decreased expression of E-cadherin together with the *de novo* expression of α-SMA was observed after incubating cells with TGF-β. Quantitation of E-cadherin and α-SMA positive cells was represented in a reciprocal manner. The inverse effect was detected after adding a neutralizing anti-TGF-β antibody, suggesting a specific response of TGF-β in this process [102].

According to previous studies, there was a dose-dependent periostin mRNA expression observed after being induced by TGF-β in mouse mesangial cells and

human collecting duct cell line [20, 22]. In animals with unilateral ureteral obstruction, there was a strong induction of TGF-β after 15 days of kidney injury [22]. The same results were also observed in a human study with different glomerulonephropathies. A positive correlation was also found between TGF-β mRNA expression and periostin mRNA expression in both glomerular and tubulointerstitial areas [20]. The relevance of periostin expression and EMT markers was also reported in both animal and human samples. In animals with some part of kidney removed, the localization of periostin was found in the distal tubule together with the disappearance of the epithelial marker (E-cadherin) [18]. The co-staining of periostin and markers involved in the EMT process (fibroblast-specific protein 1 and matrix metalloproteinase-9) was also found in tubular cells, tubular casts and interstitial cells at all time points after kidney injury [18]. The same results were also observed in mouse distal collecting tubular cells after being transfected with periostin cDNA. In contrast, after these tubular cells were transfected with SureSilencing short interfering RNA, the inverse effects of these EMT markers together with the reduction of periostin expression were found [18]. For mesenchymal markers, an increase in vimentin expression was found in animals with hypertensive nephropathy. Co-staining of periostin and vimentin was also detected in chronic allograft nephropathy patients [19].

To evaluate the association between periostin and kidney disease progression, a comparison between wild-type mice (wt mice) and mice with genetic deletion of periostin (Postn null mice) with unilateral ureteral obstruction was performed [22]. In wt mice, there was a strong induction of TGF-β and reduction of E-cadherin expression after 15 days of kidney injury. Periostin mRNA expression was increased together with vimentin mRNA expression and collagen III mRNA expression. Moreover, more fibrosis area and fibrillar collagen were found in kidney tissue from wt mice [22]. In contrast, kidney tissues from Postn null mice showed less fibrosis area and tubular dilation. Quantification results of tubulointerstitial fibrosis, tubular dilation, collagen III mRNA expression and vimentin mRNA expression were also significantly decreased in

Postn null mice compared with wt mice [22]. Lower inflammation was also reported in Postn null mice from the decline of monocyte chemoattractant protein-1 mRNA expression and macrophage infiltration within kidney tissue [22]. Moreover, the potential of periostin as a therapeutic target was also evaluated. Lower glomerulosclerosis, perivascular fibrosis, vascular hypertrophy and tubular dilation were observed after blocking periostin expression in animals with hypertensive nephropathy [22].

2.5.4 Urine Periostin Level in Kidney Injury

To date, only a few studies have focused on the potential of urine periostin measurement as a biomarker for kidney diseases. In animals with nephrectomy, no periostin was detected before kidney injury. In contrast, an increase in urine periostin excretion was found over time after kidney injury at 2 days, 2 weeks and 4 weeks with statistically significant difference compared with urine samples before nephrectomy [18]. The same was also found in kidney disease patients. Higher urine periostin levels were also detected in both proteinuric and non-proteinuric chronic kidney disease patients with statistical difference compared with healthy controls [18]. In chronic allograft nephropathy patients, the median urine periostin level in patients was significantly higher than in healthy controls. Moreover, there was a significant correlation between urine periostin level and renal functions including serum creatinine, urine protein to creatinine ratio and eGFR. The percentage of tubulointerstitial fibrosis was also significantly correlated with urine periostin in multiple regression analysis [95]. From a recent study in patients with diabetes nephropathy with different degrees of albuminuria, the median urine periostin level was increased along with the degree of albuminuria. There was a significant elevation of urine periostin level in patients with normoalbuminuria, microalbuminuria and macroalbuminuria compared with healthy controls [96]. Additionally, a positive correlation was found between urine periostin level and urine albumin to creatinine ratio. In contrast, a negative correlation was found with eGFR [96]. Other variables that also correlated with urine periostin level were age, fasting plasma glucose, hemoglobin A1C, cholesterol and low-density lipoprotein.

In multiple regression analysis, increased albuminuria, older age, and reduction of eGFR were significantly correlated with urine periostin [96]. In addition, receiver operating characteristics (ROC) analysis was also performed to find out the best cutoff level of urine periostin in patients with different chronic kidney diseases [18, 95, 96].

2.6 Periostin and Other Diseases

2.6.1 Periostin and Cancer Disease

The role of periostin in cancer disease was studied in several types of cancer diseases such as breast cancer, non-small cell lung cancer, prostate cancer and liver cancer. Immunohistochemistry analysis showed positive periostin in carcinoma cells from breast cancer patients and the breast cancer cell line, but no periostin was found from normal breast tissues [103, 104]. In addition, quantitative analysis showed an increase in periostin staining in accordance with the severity of the tumor stage. Periostin mRNA expression analysis provided the same results with a statistically significantly higher periostin level in breast cancer tissues than in normal tissues [104]. In non-small cell lung cancer patients, positive staining was also detected in bronchial basal cells and lymph node metastasis in some patients. An association between periostin expression and some clinicopathological features was reported including tumor size, lymph node metastasis, disease stage, and lymphatic invasion [105]. Moreover, poor survival was also found in patients with periostin expression [106]. The survival rate in non-small cell lung cancer patients with positive periostin was lower than in patients with negative periostin [105]. The same results were reported in patients with high periostin expression compared with low periostin expression. Additionally, periostin expression was also a prognostic factor in multivariate analysis in both non-small cell lung cancer and hepatocellular carcinoma patients [107, 108].

The role of periostin and EMT was also found in cancer disease. In the non-transformed human mammary epithelial cell line and the human breast cancer cell

line with periostin expression, there was a morphological transformation from cobblestone to an elongated fibroblast-like morphology. Immunofluorescence analysis showed that there was an increment of mesenchymal markers including N-cadherin, fibronectin, vimentin and a-SMA along with the decline of epithelial marker E-cadherin [109]. In the prostate cancer cell line with periostin transfection, epithelial marker E-cadherin mRNA expression was decreased [110]. The same results were reported in another study together with the increase of N-cadherin and fibronectin [111]. In the human lung adenocarcinoma cell line, the EMT marker was also higher in periostin-expressed cells. The mesenchymal markers N-cadherin and vimentin were increased in these cells [112]. There was a dose- and time-dependent periostin expression after being induced by TGF-β in the prostate cancer cell line. In addition, the mesenchymal markers N-cadherin and fibronectin were significantly increased after treating cells with TGF-β. The EMT process from TGF-β induction was also inhibited after decreased periostin expression, suggesting the role of periostin in the EMT process induced by TGF-β [111].

Periostin was also involved in the cell proliferation, invasion, migration and metastasis in cancer disease. After transfection with periostin in the human lung adenocarcinoma cell line, the proliferation of these cells was increased in a time-dependent pattern [112]. There was also a significant increase in cell proliferation in prostate cancer cells after being induced by periostin. Moreover, an *in vitro* invasion assay showed the increase of invasiveness in cells treated with periostin compared with the control group [110, 111]. The results from a wound healing assay demonstrated the effect of periostin for promoting cell migration. In human lung adenocarcinoma cell line, faster cell migration was observed in cells with periostin transfection than in cells without periostin transfection [112]. Additionally, there was a significant reduction of cell invasion and migration after adding the anti-periostin antibody to breast cancer cells [113]. The analysis of the metastasis effect of the anti-periostin antibody was performed in a lung metastasis model. After inoculation of breast cancer cells into a mouse foot

pad, there was an up-regulation of periostin mRNA expression in both footpads (10-fold) and lungs (100-fold) compared with normal controls. The anti-periostin antibody can also inhibit the metastasis after 3 weeks compared with the control group [113].

2.6.2 Periostin and Heart Disease

The role of periostin in heart disease was reported in both animal and human studies with different types of heart disease model. In animal hearts transfected with the periostin gene, left ventricular dilation was observed along with the abnormality of left ventricular pressure. Histology evaluation presented a decrease in cardiac myocytes diameter and increase in interstitial collagen accumulation. Up-regulation of cardiac dysfunction markers at gene level was also reported [114]. There was also an increase in periostin expression at both gene and protein level in animals with pressure overload-induced left ventricular hypertrophy. These results were decreased after 1 week of relief from pressure overload [115]. Immunohistochemistry for periostin and collagen was represented in the same manner. A significant increase in periostin staining was found in animals with left ventricular hypertrophy. More pronounced periostin and collagen were found in media, adventitia and interstitial areas. After 1 week of relief, both periostin and collagen positive areas were decreased. Additionally, a decline in periostin expression was observed after treatment in patients with end-stage heart disease [115]. Inhibition of periostin also increased the survival rate in animals with heart failure, suggesting the potential of periostin as a therapeutic target for heart failure [114].

The role of periostin in myocardial infarction was also investigated. An increase in periostin gene expression was found in the left ventricle in an animal model with myocardial infarction [116]. Positive periostin staining was observed in inflammatory and infarct regions. Cardiac fibroblast was considered as a source of periostin from periostin mRNA up-regulation in fibroblasts. Moreover, positive periostin staining was found in myocardial fibrous areas close to αv-integrin positive cardiac fibroblast in myocardial tissue from patients with acute myocardial infarction. As with the same results from a

human study, positive αv-integrin was also detected in fibroblasts from an animal model [117]. Other extracellular matrix proteins including collagen I and collagen III and fibrosis area were also increased. A positive correlation between periostin and collagen III was reported [118]. In addition, there was co-staining of periostin with other extracellular matrix protein such as collagen, laminin and fibronectin [116]. The role of periostin in cardiac healing after acute myocardial infarction was investigated in mice with genetic deletion of periostin. The survival rate was lower and an increase in mortality from cardiac rupture was reported, which suggested the vital role of periostin in the cardiac healing process [117]. Histological analysis from tissue samples showed lower number of cardiac fibroblasts in mice with genetic deletion of periostin. Moreover, the number of vimentin-positive cardiac fibroblasts, and the amount of fibronectin staining, collagen staining, and collagen cross-linking were also reduced in the infarct region. The impairment of collagen production from the absence of the periostin gene leads to the abnormality of mechanical properties. The lower number of α-SMA positive cells in the infarct area suggested the role of periostin for the recruitment of the cardiac fibroblasts. Supporting this result, an increase in α-SMA positive cells was observed after treated with recombinant periostin. Moreover, a reduction in cardiac rupture was also reported [117]. Not only cardiac tissue but also serum samples from patients with myocardial infarction were assessed for periostin level [119, 120]. At the early time point, plasma periostin level was decreased compared with healthy controls or patients with stable coronary artery disease. At 3 months, plasma periostin level was increased compared with the late time point. In multivariate analysis, acute myocardial infarction was an independent factor associated with lower plasma periostin level [119]. The occurrence of cardiovascular events including cardiovascular mortality, nonfatal stroke or transient ischemic attack, typical chest pain occurrence and re-hospitalization after six months' follow-up was higher in patients with higher serum periostin level compared with a lower level. These results demonstrated that serum periostin level may be used for the prediction of cardiovascular events in patients with myocardial infarction [120].

2.6.3 Periostin and Asthma Disease

Asthma is a chronic allergic disease involved in airway inflammation, hyper-responsiveness, airway obstruction and subepithelial fibrosis. Immune response is considered as a vital process in this allergic disease [121]. The role of periostin in the asthma process has been reported in some studies. An increase in periostin both in mRNA and protein level was observed in normal embryonic lung fibroblast cell line after being stimulated with interleukin (IL) both IL-4 and IL-13. The induction of periostin by IL-4 and IL-13 was independent of TGF-β. Supporting these results, there was a significant reduction of periostin staining from the bronchial tissue of IL-4 or IL-13 knockout mice with chronic asthma together with a decline of infiltration inflammatory cells and subepithelial fibrosis [122]. Moreover, up-regulation of periostin gene expression and protein was observed from human bronchial epithelial cells stimulated by IL-13. The expression of periostin was also detected in bronchial epithelial cells from asthmatic patients [123]. The subepithelial region was thicker and more positive for periostin than in patients without asthma [122]. A positive correlation between periostin gene expression in epithelial brushings and the thickness of subepithelial fibrosis was found in biopsy samples from asthmatic patients, suggesting epithelial cells as a source of periostin secretion which related to the subepithelial fibrosis process [123]. The relevance of periostin and collagen I was also reported which involved the EMT process. There was an increase in both collagen I gene and protein level together with a loss of epithelial marker E-cadherin and more expression of the mesenchymal markers vimentin and α-SMA in bronchial epithelial cell line transfected with human recombinant periostin expression vector. The characteristics of epithelial cells were changed to an elongated shape by reducing the connection between each other [123]. Moreover, marked up-regulation of MMP-2 and MMP-9 gene expression was found from bronchial epithelial cells transfected with periostin through the activation of TGF-β. Supporting these results, the TGF-β1 and collagen gene expression was increased in human bronchial epithelial cells after being treated with recombinant periostin. The loss of E-cadherin

expression in accordance with more expression of the mesenchymal marker vimentin was also observed [123].

According to previous studies, serum periostin was considered as a novel biomarker related to eosinophilic inflammation in asthmatic patients. The serum periostin level was higher in asthmatic patients than in healthy controls [124, 125]. A higher serum periostin level was also observed in “eosinophil-high” patients compared with “eosinophil-low” patients. An increase in serum periostin was detected along with a higher score of eosinophil from sputum and tissue evaluation [126]. Moreover, logistic regression model analysis with the possible predictor of eosinophil status including age, sex, body mass index, blood eosinophil numbers, serum IgE, fraction of exhaled nitric oxide and serum periostin levels found that serum periostin was the most significant predictor for airway eosinophil status. In addition, receiver operating characteristic analysis of serum periostin suggested the potential of serum periostin as a marker for airway inflammation [126]. A decline of pulmonary function and higher peripheral eosinophil counts were observed in patients with high serum periostin compared with low serum periostin. A positive correlation was also found between serum periostin level and peripheral blood eosinophilia. Moreover, significantly higher IL-4 and IL-13 were reported in the high serum periostin group compared with the low serum periostin group, suggesting the potential of serum periostin as a non-invasive measurement related to the inflammation of asthma disease [125].

CHAPTER III

RESEARCH METHODOLOGY

3.1 Study Samples

This study was conducted from April 2013 to February 2015 at the Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand. It was approved by the institutional review boards and ethics review committees of the Royal Thai Army Medical Department, Phramongkutklao Hospital and College of Medicine, Bangkok, Thailand (No. 489/2556 and 1168/2556) (Appendix A). Lupus nephritis or IgA nephropathy patients who had a diagnosis confirmed by a pathologist at Phramongkutklao Hospital were included in this study with the following criteria:

Inclusion criteria

1. Age $\geq$ 18 years.
2. Patients who had indications for renal biopsy and were diagnosed with lupus nephritis or IgA nephropathy according to the definitions confirmed by a pathologist.
3. More than or equal to 3 glomeruli were obtained from the biopsy.

Exclusion criteria

1. Patients with urinary tract obstruction, urinary tract infection and kidney transplant.
2. Patients with cancer diseases.
3. Patients with asthma.

4. Patients with advanced heart diseases.
5. Pregnancy and lactation.

3.2 Sample Size Calculation

The primary objective of this study was to investigate the correlation between periostin staining and renal pathology in patients with lupus nephritis and IgA nephropathy. The sample size for the correlation study was calculated as follows:

$$N = \left[\frac{2Z_{\alpha/2}}{Z_u - Z_l}\right]^2 + 3$$

Define $\alpha = 0.05$

$Z_{\alpha/2} =$ 1.96 (Two tail)

$$Z_u = 0.5 \times \ln\left[\frac{1+\rho_u}{1-\rho_u}\right]$$

$$Z_l = 0.5 \times \ln\left[\frac{1+\rho_l}{1-\rho_l}\right]$$

$\rho =$ Population correlation coefficient

$\rho_u =$ Upper limit of population correlation

$\rho_l =$ Lower limit of population correlation

According to the results from the study of Sen *et al.* in 2011 to find out the correlation between the periostin positive area in kidney tissues and eGFR [20].

Therefore

$\rho =$ Population correlation coefficient = -0.695

$\rho_u =$ Upper limit of population correlation = -0.834

$\rho_l =$ Lower limit of population correlation = -0.556

$$Z_u = 0.5 \times \ln\left[\frac{1+\rho_u}{1-\rho_u}\right] = -1.201$$

$$Z_l = 0.5 \times \ln\left[\frac{1+\rho_l}{1-\rho_l}\right] = -0.627$$

Sample size calculation

$$N = \left[\frac{2\ (1.96)}{-1.201 + 0.627}\right]^2 + 3$$

$$= 49.64$$

Therefore, the total sample needed for this study was 50 patients.

Controls

Five normal kidney tissue sections from renal cell carcinoma patients confirmed by a renal pathologist were used as controlled kidney tissues. Urine and serum samples from 50 healthy controls aged over or equal to 18 years, without any underlying diseases and have normal renal function were used for periostin level comparison among patients.

3.3 Data Collection

Patients who met the inclusion and exclusion criteria were included in this research. An information sheet and informed consent were obtained before collecting each patient's data. The consent form included data about the objectives of the

research, inclusion and exclusion criteria, procedures, time duration of the procedures, discomforts and risks, potential benefits, costs and compensation for participation, research funding and contact information. Participants were informed that all data were collected for scientific research only and kept confidential. All participants were interviewed for collecting demographic data. Laboratory testing and renal parameters including serum creatinine, blood urea nitrogen, serum albumin and urine protein to creatinine ratio were reviewed from each patient's record. Glomerular filtration rate was calculated from the CKD-EPI equations as follows [46].

For women with serum creatinine ≤ 0.7 mg/dl

$$GFR\ (ml/min/1.73\ m^2) = (Scr/0.7)^{-0.329} \times (0.993)^{Age}\ (\times 166 \text{ if black; } \times 144 \text{ if white or other})$$

For women with serum creatinine > 0.7 mg/dl

$$GFR\ (ml/min/1.73\ m^2) = (Scr/0.7)^{-1.209} \times (0.993)^{Age}\ (\times 166 \text{ if black; } \times 144 \text{ if white or other})$$

For men with serum creatinine ≤ 0.9 mg/dl

$$GFR\ (ml/min/1.73\ m^2) = (Scr/0.9)^{-0.411} \times (0.993)^{Age}\ (\times 163 \text{ if black; } \times 141 \text{ if white or other})$$

For men with serum creatinine > 0.9 mg/dl

$$GFR\ (ml/min/1.73\ m^2) = (Scr/0.9)^{-1.209} \times (0.993)^{Age}\ (\times 163 \text{ if black; } \times 141 \text{ if white or other})$$

3.4 Sample Collection and Measurement

3.4.1 Kidney Tissue Sample for Renal Pathology Evaluation and Periostin Staining

Kidney tissues were obtained from patients who had a definite diagnosis of lupus nephritis or IgA nephropathy by a pathologist and normal kidney tissue sections from renal cell carcinoma patients which were confirmed by a renal pathologist. Three-micrometer-thick sections of paraffin-embedded kidney were stained with

hematoxylin and eosin (H&E), Masson's trichrome, Periodic Acid-Schiff (only in IgA nephropathy patients) and immunohistochemistry for periostin (Appendix B). Immunohistochemistry was used for demonstrating the presence and location of periostin in kidney tissue by using the Bench Mark XT automated slide preparation system (Ventana, USA). Three-micron sections of formalin-fixed paraffin-embedded tissue were deparaffinized and rehydrated. Rabbit polyclonal to periostin was added as primary antibody (Abcam, Cambridge, UK). Antibody conjugated with horseradish peroxidase was used as secondary antibody. Reactivity was detected using diaminobenzidine reagent and then counterstained with hematoxylin II. The uterus and ovary were used as positive and negative internal controls, respectively. Kidney tissue-stained slides were then scanned with a Panoramic MIDI Slide Scanner (3DHISTECH, Hungary) before histology evaluation. Positive staining was detected as a brown coloration of the tissues and periostin staining was evaluated by a renal pathologist.

Renal pathology within kidney tissue was assessed by a renal pathologist. The activity index score and chronicity index score were calculated from summing the score in both glomerular and tubulointerstitial abnormalities as shown in Table 5 [28]. Patients with low active disease were patients with activity index score lower than 8. Patients with low chronic disease were patients with chronicity index score lower than 4. A higher activity index score or chronicity index score represented a higher active or chronic of disease, respectively. Patients with a higher activity score should receive more immunosuppressive agents. On the other hand, a higher chronicity score is less likely to respond to immunosuppressive agents [26]. Periostin staining was also evaluated in glomerular, interstitial, vascular and tubular abnormalities. Scores were graded from 0 to 5 as a percentage of the affected area with positive periostin. Intensity was graded from 0 to 3 [127]. The periostin staining score in each histological feature was calculated by score multiplied by intensity. The total periostin staining score was calculated from the summation of the periostin staining score in each pathological abnormality as shown in

Table 6. A higher periostin staining score represented a higher periostin expression in kidney tissues.

Table 5 Evaluation of activity index and chronicity index [28]

Activity index	Activity score	Chronicity index	Chronicity score
Glomerular abnormalities		**Glomerular abnormalities**	
Glomerular cell proliferation	0-3	Glomerular sclerosis	0-3
Fibrinoid necrosis or karyorrhexis	0-6	Fibrous crescents	0-3
Cellular crescents	0-6		
Hyaline thrombi or wire loops[a]	0-3		
Glomerular leukocyte infiltration[a]	0-3		
Tubulointerstitial abnormality		**Tubulointerstitial abnormalities**	
Interstitial inflammation[a]	0-3	Interstitial fibrosis[a]	0-3
		Tubular atrophy[a]	0-3
Total score	**0-24**	**Total score**	**0-12**
Scores were graded from 0 to 3 as percent of glomeruli and interstitium affected area: 0 = absent; 1= less than 25% ; 2 = 25% to 50%; 3 = more than 50% [a]0 = absent; 1= mild ; 2 = moderate; 3 = extensive Fibrinoid necrosis and cellular crescents are weighted double.			

Table 6 Evaluation of periostin staining score [127]

Periostin positive staining	Score[a] (0-5)	Intensity[b] (0-3)	Periostin staining score[c] (0-15)
Periglomerular staining	0-5	0-3	0-15
Mesangial staining			
Fibrocellular crescent			
Fibrous crescent			
Segmental sclerosis			
Global sclerosis			
Interstitial fibrosis			
Vascular fibrosis			
Tubular epithelial cell staining			
Tubular atrophy			
Tubular cell cast			
Total periostin staining score	**0-55**	**0-33**	**0-165**

[a]Score were graded from 0 to 5 as percent of affected glomeruli, interstitial, vascular and tubular area with positive periostin:
0 = absent; 1= 1% ; 2 = 2% to 10% ; 3 = 11% to 33%; 4 = 34% to 66%; 5 = 67% to 100%

[b]Intensity was graded from 0 to 3.

[c]Periostin staining score in each histological feature was calculated by score multiplied by intensity.

3.4.2 Kidney Tissue Sample for Periostin mRNA Expression Evaluation

There were 13 kidney tissue samples from patients and 5 control kidney tissues could be obtained for periostin mRNA expression analysis by quantitative real-time polymerase chain reaction (PCR). Additionally, a principal inducer of EMT process,

TGF-β mRNA expression was also evaluated with the same method. Total RNA was extracted from patients and controlled kidney tissues with a commercial kit (RNeasy Mini kit; Qiagen Inc, Chatworth, CA). Total RNA was then converted to cDNA by reverse transcriptase (MonsterScript 1st-Strand cDNA Synthesis Kit; Qiagen Inc, Chatworth, CA) and followed by PCR amplification of the cDNA (All-in-One™ qPCR Mix; GeneCopoeia, Rockville, MD). Real-time PCR was performed by using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) (Appendix C).

3.4.3 Urine Sample for Periostin Measurement

Urine samples were collected (at least 25 ml) from 50 patients on the same day as the kidney biopsy and 50 healthy controls. They were centrifuged to remove sediment and frozen in aliquots at -80 °C until assay. Urine periostin was measured by enzyme-linked immunosorbent assay (ELISA) (Appendix D) [18]. A polyclonal antibody specific for periostin was pre-coated onto a microplate. Periostin standards and urine samples were added into the wells. Polyclonal antibody specific for periostin was added as primary antibody. Horseradish peroxidase conjugated antibody was used as secondary antibody. Substrate solution was added and color developed. After adding stop solutions, the intensity was measured at 450 nm. A log-transformed standard curve was generated and the urine periostin concentrations were calculated. The urine periostin level (ng/mg creatinine) was further calculated by correction with urine creatinine. Urine creatinine was measured by using standard method.

3.4.4 Serum Sample for Periostin Measurement

Five centimeters of venous blood were obtained from 50 patients on the same day as the kidney biopsy and 50 healthy controls. They were centrifuged to collect serum and frozen in aliquots at -80 °C until assay. Serum periostin was measured by ELISA (Appendix E). A polyclonal antibody specific for periostin was pre-coated onto a microplate. Periostin standards and serum samples (dilute 1:50) were added into the

wells. Polyclonal antibody specific for periostin was added as primary antibody. Horseradish peroxidase conjugated antibody was used as secondary antibody. Substrate solution was added and color developed. After adding stop solutions, the intensity was measured at 450 nm. A log-transformed standard curve was generated and the serum periostin concentrations were calculated.

3.4.5 Clinical Response to Therapy

After 6 months of treatment according to ACR guideline for lupus nephritis or KDIGO guideline for IgA nephropathy patients, patients were assessed for treatment response [26, 36]. Patients with complete response or partial response were classified as “patients with response to therapy.” Patients with deterioration were classified as “patients with non-response to therapy.” Definitions of response to therapy were described as follows (adapted from KDIGO guideline) [36].

1. Complete response

Return of serum creatinine to previous baseline, plus a decline in the urine protein to creatinine ratio to < 500 mg/g (< 50 mg/mmol).

2. Partial response

Stabilization (±25%), or improvement of serum creatinine, but not to normal, plus a ≥ 50% decrease in urine protein to creatinine ratio. If there is nephrotic-range proteinuria (urine protein to creatinine ratio ≥ 3,000 mg/g [≥ 300 mg/mmol]), improvement requires a ≥ 50% reduction in urine protein to creatinine ratio, and a urine protein to creatinine ratio < 3,000 mg/g [< 300 mg/mmol].

3. Deterioration

A sustained 25% increase in serum creatinine is widely used. Other responses that do not meet the complete or partial response definitions are also included in this type of response.

Urine samples were also collected from patients for urine periostin measurement by ELISA. Urine periostin level after 6 months of treatment from patients was compared with urine periostin level at baseline in both patients with response and non-response to therapy. .

3.5 Statistical Analysis

Statistical analysis was performed using the SPSS statistical software package version 18. Characteristics of patients were presented as percentages for describing nominal and ordinal data. Mean ± SD or median with interquartile ranges was reported for continuous variables depending on a normality test. Spearman rank correlation was used to find out the correlation between periostin level and other variables. The Mann-Whitney U test was used for comparing two independent sample groups. The Wilcoxon Signed Ranks Test was used for comparing two related sample groups. In this study, a p-value less than 0.05 was considered statistically significant. Receiver operating characteristics analysis was generated to find the best cutoff values of urine periostin level for distinguishing healthy controls from patients. The overall statistical testing in this study is shown in Table 7.

Table 7 Statistical testing in this study

Hypotheses	Statistical testing
There was a correlation between periostin staining and renal pathology	Spearman rank correlation
There was a correlation between urine periostin level and renal pathology	Spearman rank correlation
There was a correlation between serum periostin level and renal pathology	Spearman rank correlation
The median of variables between patients with high periostin staining score was different from patients with low periostin staining score	Mann-Whitney U test
The median of periostin mRNA expression in patients was different from control	Mann-Whitney U test
The median of urine periostin level in patients was different from healthy control	Mann-Whitney U test
The median of serum periostin level in patients was different from healthy control	Mann-Whitney U test
There was a correlation between periostin staining and other variables (including renal parameters)	Spearman rank correlation
There was a correlation between urine periostin level and other variables (including renal parameters)	Spearman rank correlation
There was a correlation between serum periostin level and other variables (including renal parameters)	Spearman rank correlation
The median of variables in patients with response to therapy was different from patients with non- response to therapy	Mann-Whitney U test
The median of urine periostin level after 6 months of treatment was different from urine periostin level at biopsy date	Wilcoxon Signed Ranks Test

3.6 Ethical Consideration

All participants were fully informed about the objectives and the process of the study by information sheet before deciding to participate in the study. The researcher did not attempt to force the patients to decide to be participants and informed them that their decision would not affect their treatment or service. Written informed consent was obtained before collecting the data from participants. Only individuals who agreed to participate were included in this study. The participants' information was kept confidential. The data were analyzed and reported in general. Although kidney biopsy is an invasive procedure, it was performed by nephrologists in patients who had indications only. All patients were observed in hospital at least one day after biopsy to make sure that no complications had occurred.

CHAPTER IV

RESULTS

4.1 Participants' Demographic Data

A total of 50 patients were included in this study. Most of the patients were female with an average age of 32 years. There were 37 patients diagnosed with lupus nephritis. Most patients were classified into class III and IV according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS). Systemic lupus erythematosus was a common comorbid disease found in 87% of lupus nephritis patients. For IgA nephropathy, hypertension was the most common comorbid disease found in 54% of patients. According to the Oxford classification, segmental glomerulosclerosis and endocapillary hypercellularity were the most common pathological features reported in most IgA nephropathy patients. Renal function impairment was found in both lupus nephritis and IgA nephropathy patients. Overall clinical characteristic data from controls and patients are shown in Table 8.

Table 8 Clinical characteristic data

Characteristic data	Mean ± SD [Range]			
	Healthy Controls (n=50)	Overall Patients (n=50)	LN Patients (n=37)	IgAN Patients (n=13)
Gender (n, %)				
Female	17 (34%)	41 (82%)	34 (92%)	7 (54%)
Male	33 (66%)	9 (18%)	3 (8%)	6 (46%)
Age (years)	30.1 ± 9.5 [21.0-58.0]	31.8 ± 11.8 [18.0-59.0]	29.8 ± 10.4 [18.0-58.0]	37.7 ± 14.0 [18.0-59.0]
Body weight (kg)	70.8 ± 13.4 [41.0-103.9]	57.0 ± 13.3 [30.0-85.0]	54.8 ± 13.2 [30.0-85.0]	63.2 ± 11.9 [45.0-85.0]
Height (cm)	167.7 ± 8.2 [150.0-180.0]	158.7 ± 10.0 [123.0-182.0]	157.4 ± 10.1 [123.0-180.0]	162.3 ± 8.7 [150.0-182.0]
Body mass index (kg/m^2)	25.0 ± 3.6 [17.4-31.9]	22.6 ± 4.5 [15.5-34.1]	22.1 ± 4.8 [15.5-34.1]	23.9 ± 3.3 [17.6-29.7]
Renal diseases (n, %)	-			-
LN	-	37 (74%)	37 (100%)	
ISN/RPS class (n, %)				
I			0 (0%)	
II			1 (3%)	
III			12 (32%)	
IV			12 (32%)	
V			1 (3%)	
VI			0(0%)	
Mix classification (n, %)				
III + V			5 (14%)	
IV + V			6 (16%)	

Table 8 Clinical characteristic data (cont.)

Characteristic data	Mean ± SD [Range]			
	Healthy Controls (n=50)	Overall Patients (n=50)	LN Patients (n=37)	IgAN Patients (n=13)
IgA nephropathy Oxford classification (n,%)	-	13 (26%)	-	13 (100%)
Mesangial hypercellularity				
M0				8 (62%)
M1				5 (38%)
Segmental glomerulosclerosis				
S0				5 (38%)
S1				8 (62%)
Endocapillary hypercellularity				
E0				2 (15%)
E1				11 (85%)
Tubular atrophy /interstitial fibrosis				
T0				8 (62%)
T1				2 (15%)
T2				3 (23%)
Comorbid diseases (n, %)				
SLE	-	32 (64%)	32 (87%)	0 (0%)
Hypertension	-	20 (40%)	13 (35%)	7 (54%)
Dyslipidemia	-	9 (18%)	5 (14%)	4 (31%)

Table 8 Clinical characteristic data (cont.)

Characteristic data	Mean ± SD [Range]			
	Healthy Controls (n=50)	Overall Patients (n=50)	LN Patients (n=37)	IgAN Patients (n=13)
Systolic blood pressure (mmHg)	124.0 ± 17.3 [87.0-164.0]	135.1 ± 20.0 [93.0-185.0]	135.8 ± 21.3 [93.0-185.0]	133.1 ± 16.3 [109.0-165.0]
Diastolic blood pressure (mmHg)	76.1 ± 11.0 [51.0-105.0]	83.4 ± 15.8 [46.0-120.0]	84.8 ± 15.5 [56.0-120.0]	79.4 ± 16.6 [46.0-118.0]
Renal parameters				
Serum creatinine (mg/dl) [a]	0.8 (0.7, 0.9) [0.5-0.9]	0.8 (0.7, 1.3) [0.5-5.2]	0.8 (0.7, 0.9) [0.5-3.3]	1.5 (0.8, 2.3) [0.6-5.2]
Blood urea nitrogen (mg/dl) [a]	11.6 (9.1, 12.5) [4.9-15.4]	17.5 (12.8, 25.7) [6.3-93.3]	19.2 (12.8, 25.7) [6.3-93.3]	16.9 (13.1, 24.4) [9.2-82.1]
Serum albumin (g/dl)	-	3.2 ± 0.7 [1.6-4.3]	3.1 ± 0.6 [1.6-4.3]	3.7 ± 0.7 [2.1-4.3]
Urine protein to creatinine ratio [a]	-	2.19 (0.89, 4.48) [0.07-7.99]	2.58 (0.78, 4.55) [0.07-7.93]	1.37 (1.16, 2.30) [0.11-7.99]
eGFR (ml/min/1.73 m^2)[¶]	119.69 ± 10.14 [96.70-142.72]	87.67 ± 36.02 [13.32-141.75]	96.42 ± 33.64 [25.29-141.75]	62.78 ± 31.55 [13.32-106.29]

[a] Data was reported as Median (Q1, Q3) [Range]

[¶] eGFR: estimated glomerular filtration rate

4.2 Renal Pathology Evaluation

4.2.1 Activity and Chronicity Index Score

Activity index score and chronicity index score were assessed by a renal pathologist in kidney tissue from patients and controls (Table 9-10). For controls, no activity index and chronicity index were observed. Therefore, total activity index score and total chronicity index score were 0 (0, 0). For the activity index score, glomerular cell proliferation and hyaline thrombi or wire loop were the most common findings in overall patients with a median score of 2 (1, 3) and 1 (0, 1), respectively. Glomerular leukocyte infiltration and cellular crescents were also found in 32% and 28% of patients, respectively. The same tendency was also reported in subgroup analysis of LN and IgA nephropathy patients with median total activity index scores of 4 (2, 6) and 3 (2, 5), respectively. For the chronicity index score, interstitial fibrosis and tubular atrophy were the most common findings found in 72% of overall patients with the same median score of 1 (0, 1). Half of the overall patients also presented glomerular sclerosis. The same was reported in both LN and IgA nephropathy patients with median total chronicity index scores of 2 (0, 4) and 4 (3, 7), respectively.

Table 9 Activity index score from overall patients, LN and IgAN patients

Activity index	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	N[a] (%)	Score Median (Q1, Q3) [Range]	N[a] (%)	Score Median (Q1, Q3) [Range]	N[a] (%)	Score Median (Q1, Q3) [Range]
Glomerular abnormalities						
Glomerular cell proliferation	46 (92)	2 (1, 3) [0-3]	35 (95)	2 (1, 3) [0-3]	11 (85)	2 (1, 2) [0-3]
Fibrinoid necrosis or karyorrhexis	3 (6)	0 (0, 0) [0-2]	3 (8)	0 (0, 0) [0-2]	0 (0)	0 (0, 0) [0-0]
Cellular crescents	14 (28)	0 (0, 2) [0-4]	9 (24)	0 (0, 0) [0-4]	5 (39)	0 (0, 2) [0-4]
Hyaline thrombi or wire loop	29 (58)	1 (0, 1) [0-3]	21 (57)	1 (0, 1) [0-3]	8 (62)	1 (0, 1) [0-2]
Glomerular leukocyte infiltration	16 (32)	0 (0, 1) [0-2]	12 (32)	0 (0, 1) [0-2]	4 (31)	0 (0, 1) [0-1]
Tubulointerstitial abnormality						
Interstitial inflammation	5 (10)	0 (0, 0) [0-1]	3 (8)	0 (0, 0) [0-1]	2 (15)	0 (0, 0) [0-1]
Total activity index score		4 (2, 6) [0-11]		4 (2, 6) [0-11]		3 (2, 5) [1-9]

[a]Number of patients with abnormal histological features.

Table 10 Chronicity index score from overall patients, LN and IgAN patients

Chronicity index	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	N[a] (%)	Score Median (Q1, Q3) [Range]	N[a] (%)	Score Median (Q1, Q3) [Range]	N[a] (%)	Score Median (Q1, Q3) [Range]
Glomerular abnormalities						
Glomerular sclerosis	26 (52)	1 (0, 1) [0-3]	17 (46)	0 (0, 1) [0-2]	9 (69)	1 (0, 2) [0-3]
Segmental sclerosis	17 (34)	0 (0, 1) [0-2]	9 (24)	0 (0, 0) [0-2]	8 (62)	1 (0, 1) [0-2]
Global sclerosis	18 (36)	0 (0, 1) [0-2]	12 (32)	0 (0, 1) [0-2]	6 (46)	0 (0, 1) [0-2]
Fibrous crescents	11 (22)	0 (0, 0) [0-3]	7 (19)	0 (0, 0) [0-2]	4 (31)	0 (0, 1) [0-3]
Tubulointerstitial abnormalities						
Interstitial fibrosis	36 (72)	1 (0, 1) [0-3]	24 (65)	1 (0, 1) [0-3]	12 (92)	1 (1, 2) [0-3]
Tubular atrophy	36 (72)	1 (0, 1) [0-3]	24 (65)	1 (0, 1) [0-3]	12 (92)	1 (1, 2) [0-3]
Total chronicity index score		3 (2, 4) [0-12]		2 (0, 4) [0-8]		4 (3, 7) [0-12]

[a]Number of patients with abnormal histological features.

4.2.2 Periostin Staining Score

Periostin staining score was also evaluated by a renal pathologist in kidney tissue from both patients and controls (Appendix F). No periostin was detected in control kidney tissues (Figure 1). In contrast, periostin staining was detected in glomerular, interstitial, tubular and vascular areas from patients' kidney tissues. In LN patients, periostin was detected in periglomerular fibrosis and sclerosed glomeruli. There was also positive periostin in blood vessels (Figure 2-3). In IgA nephropathy, periostin was found in areas with periglomerular fibrosis, fibrous crescent and global glomerular sclerosis. Non-atrophic and atrophic tubular epithelial cells as well as tubular casts were also positive for periostin staining (Figure 4-5). According to periostin staining analysis, periglomerular staining was found in 56% of overall patients with median periostin score of 3 (0, 9). There was also positive periostin in areas with interstitial fibrosis and sclerosed glomeruli reported in half of patients. Additionally, the most common area with positive periostin was tubular including tubular epithelial cells, tubular cell casts and tubular atrophy with median scores of 2 (0, 6), 2 (0, 5) and 0 (0, 2), respectively. The total periostin staining score was 19.3 (3, 32). Subgroup analysis in LN patients found that tubular epithelial cells, tubular atrophy, periglomerular staining and interstitial fibrosis were also positive for periostin. The total periostin staining score was 16 (2, 30.5). For IgA nephropathy patients, positive periostin staining in periglomeruli, global sclerosis, interstitial fibrosis, vascular fibrosis, tubular epithelial cells and tubular cell casts was observed in more than half of the patients. The total periostin staining score was 22 (12.5, 32.5). Overall periostin staining score is shown in Table 11.

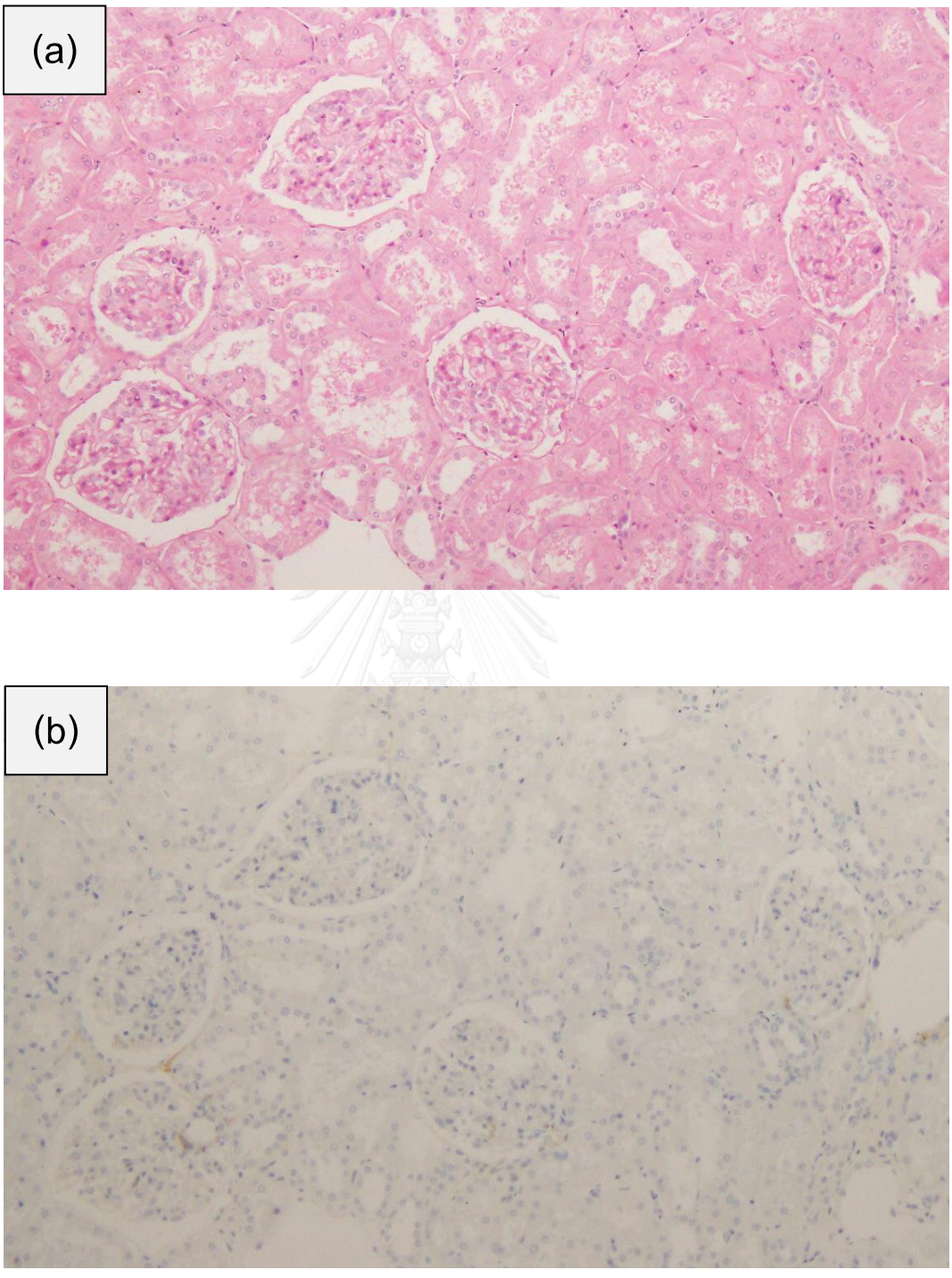

Figure 1 Normal kidney tissue section from control kidney sample. (a) H&E staining (Original magnification: x100) (b) Periostin immunostaining was not presented in control kidney tissue (Original magnification: x100).

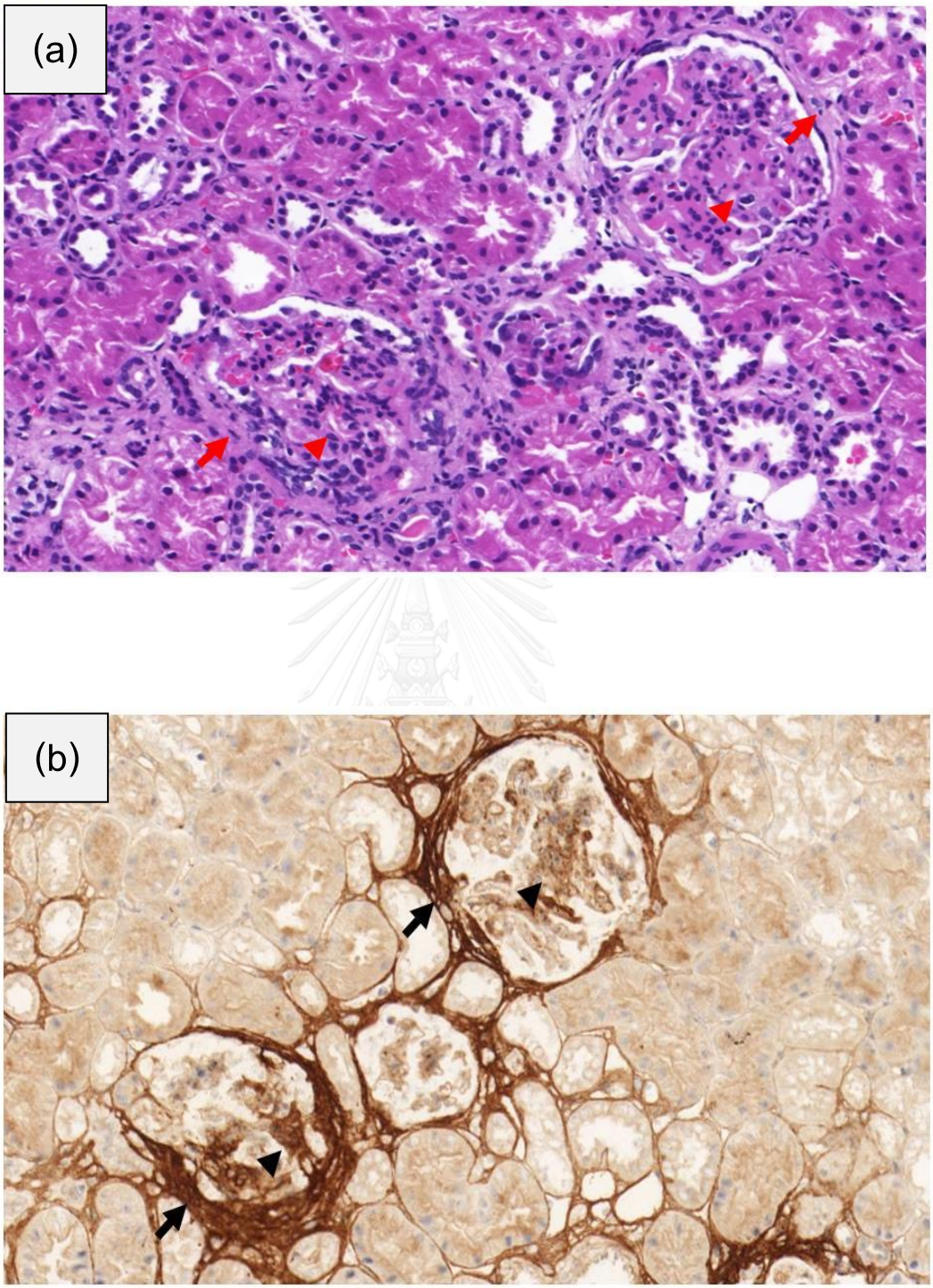

Figure 2 Renal biopsy from patient A with lupus nephritis; (a) H&E staining showed segmental sclerosis (arrow heads) and mild periglomerular fibrosis (arrows) (Original magnification: x200). (b) Periostin immunostaining presented in areas with periglomerular fibrosis (arrows) and glomerular sclerosis (arrow heads) (Original magnification: x200).

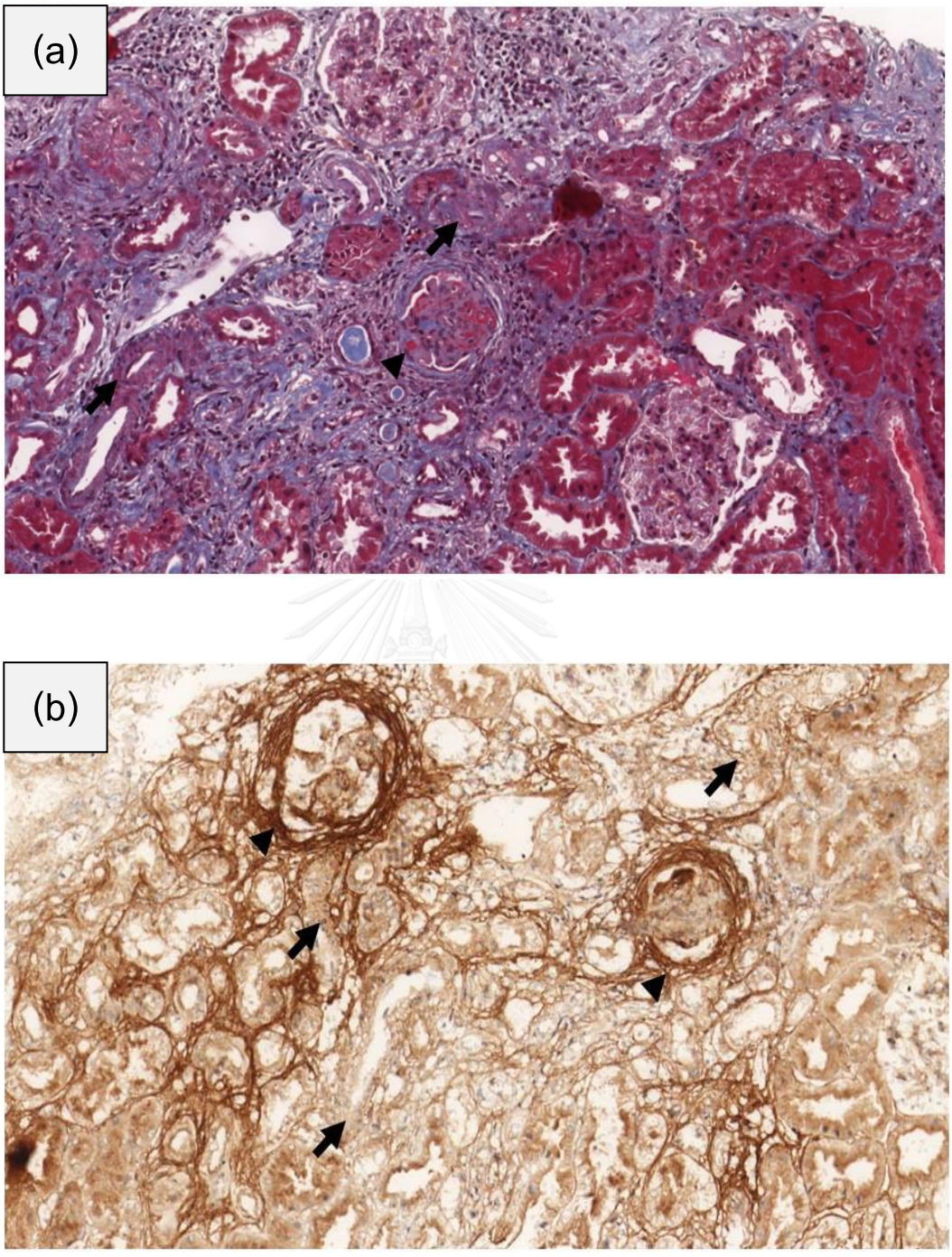

Figure 3 Renal biopsy from patient B with lupus nephritis; (a) Masson's trichrome staining showed periglomerular fibrosis (arrowhead) and vascular sclerosis (arrows) (Original magnification: x150). (b) Periostin immunostaining presented within periglomerular areas (arrowheads) and blood vessels (arrows) (Original magnification: x200).

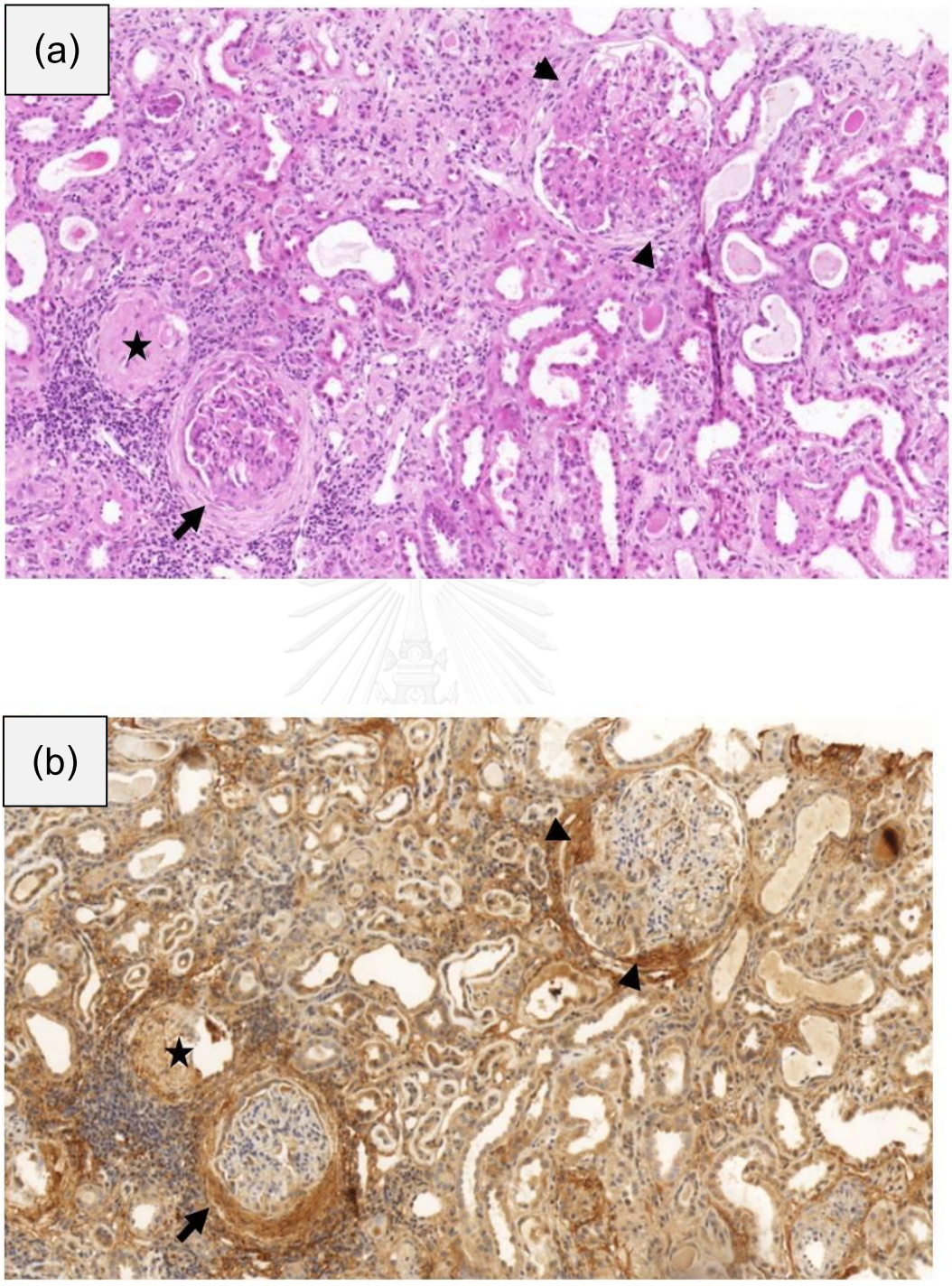

Figure 4 Renal biopsy from patient C with IgA nephropathy; (a) H&E staining showed periglomerular fibrosis (arrow), fibrous crescent (arrow heads) and global glomerular sclerosis (star) (Original magnification: x100). (b) Periostin immunostaining presented in areas with periglomerular fibrosis (arrow), fibrous crescent (arrow heads) and global glomerular sclerosis (star) (Original magnification: x100).

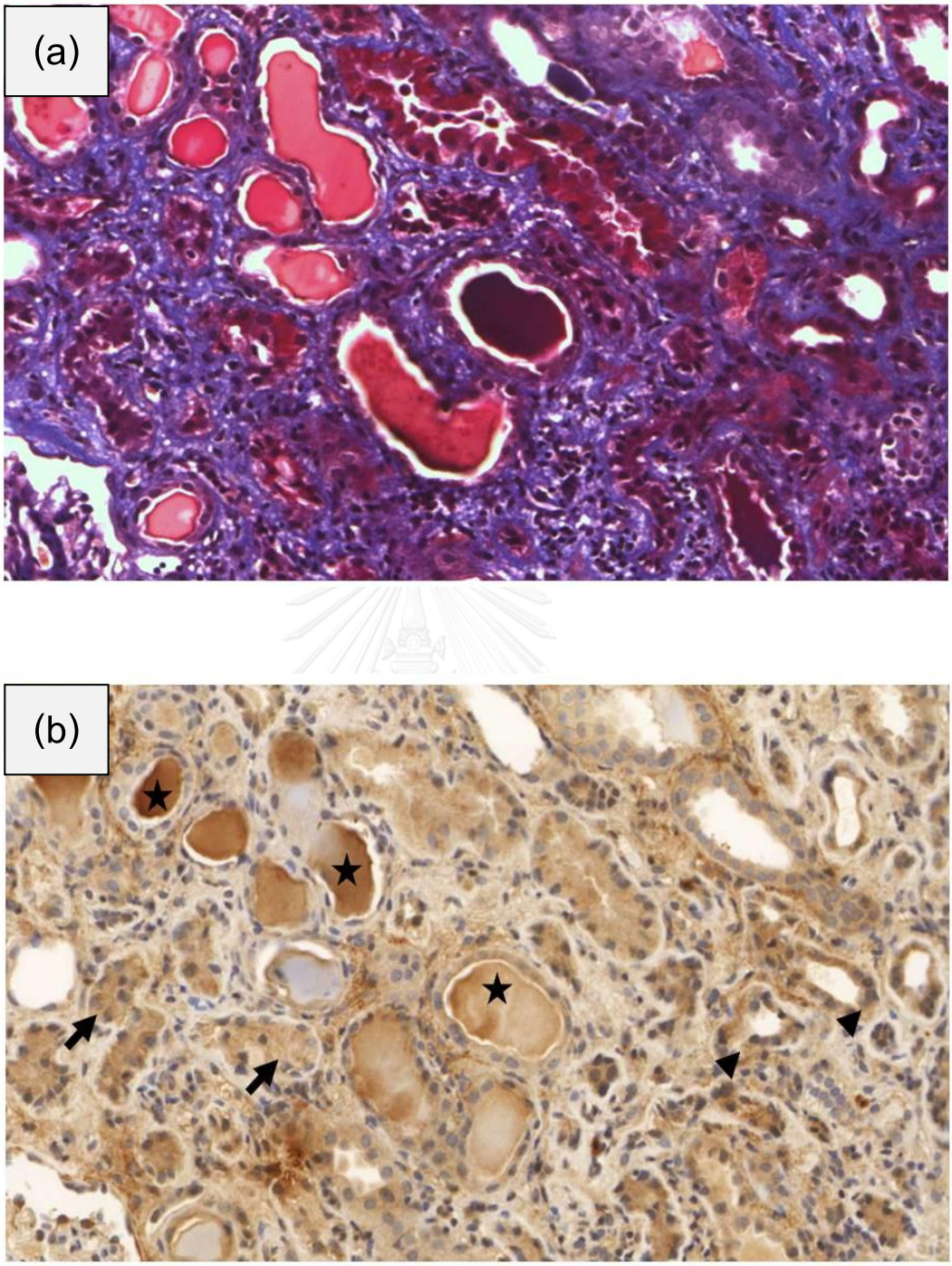

Figure 5 Renal biopsy from patient C with IgA nehropathy; (a) Masson's trichrome staining showed interstitial fibrosis and tubular atrophy with intratubular casts (Original magnification: x200). (b) Periostin immunostaining presented within non-atrophic (arrows) and atrophic (arrow heads) tubular epithelial cells as well as intratubular casts (stars) (Original magnification: x200).

Table 11 Periostin staining score from overall patients, LN and IgAN patients

Periostin staining	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	N [a] (%)	Periostin staining score Median (Q1, Q3) [Range]	N [a] (%)	Periostin staining score Median (Q1, Q3) [Range]	N [a] (%)	Periostin staining score Median (Q1, Q3) [Range]
Periglomerular staining	28 (56)	3 (0, 9) [0-15]	20 (54)	2 (0, 8) [0-15]	8 (62)	6 (0, 9) [0-15]
Mesangial staining	6 (12)	0 (0, 0) [0-8]	4 (11)	0 (0, 0) [0-8]	2 (15)	0 (0, 0) [0-3]
Fibrocellular crescent staining	11 (22)	0 (0, 0) [0-9]	8 (22)	0 (0, 0) [0-9]	3 (23)	0 (0, 0) [0-9]
Fibrous crescent staining	3 (6)	0 (0, 0) [0-12]	0 (0)	0 (0, 0) [0-0]	3 (23)	0 (0, 0) [0-12]
Segmental sclerosis staining	11 (22)	0 (0, 0) [0-9]	6 (16)	0 (0, 0) [0-9]	5 (39)	0 (0, 3) [0-6]
Global sclerosis staining	14 (28)	0 (0, 2) [0-6]	7 (19)	0 (0, 0) [0-6]	7 (54)	2 (0, 3) [0-4]
Interstitial fibrosis staining	24 (48)	0 (0, 4) [0-6]	16 (43)	0 (0, 4) [0-6]	8 (62)	2 (0, 4) [0-6]
Vascular fibrosis staining	17 (34)	0 (0, 5) [0-15]	10 (27)	0 (0, 2) [0-15]	7 (54)	4 (0, 5) [0-10]
Tubular epithelial cell staining	32 (64)	2 (0, 6) [0-10]	22 (60)	2 (0, 5) [0-10]	10 (77)	5 (2, 6) [0-10]
Tubular atrophy staining	20 (40)	0 (0, 2) [0-8]	14 (38)	0 (0, 2) [0-6]	6 (46)	0 (0, 2) [0-8]
Tubular cell cast staining	26 (52)	2 (0, 5) [0-10.5]	17 (46)	0 (0, 5) [0-10]	9 (69)	5 (0, 5.5) [0-10.5]
Total periostin staining score		19.3 (3, 32) [0-65]		16.0 (2, 30.5) [0-47]		22.0 (12.5, 32.5) [2-65]

[a]Number of patients with periostin staining in each abnormal histological features.

4.2.3 Correlation of Total Periostin Staining Score

The correlation between periostin staining score and renal pathology or other variables including renal parameters was also evaluated (Table 12-14). No correlation was observed between total periostin staining score and activity index score. In contrast, there was a significant correlation between total periostin staining score and chronicity index score ($r = 0.527$, p-value < 0.001). A positive correlation was also found between total periostin staining score and renal pathology score within the chronicity index including segmental sclerosis ($r = 0.361$, p-value $= 0.010$), fibrous crescent ($r = 0.339$, p-value $= 0.016$), interstitial fibrosis ($r = 0.416$, p-value $= 0.003$) and tubular atrophy ($r = 0.416$, p-value $= 0.003$). There was a significant correlation between total periostin staining score and interstitial fibrosis ($r = 0.504$, p-value $= 0.001$) or tubular atrophy ($r = 0.504$, p-value $= 0.001$) in LN patients. In contrast, only fibrous crescent correlated with the total periostin staining score ($r = 0.700$, p-value $= 0.008$) in IgA nephropathy patients (Table 12). Taking into consideration each histopathological chronicity index features and periostin staining in those features, a significant positive correlation was also found (Table 13). In addition, periostin staining also correlated with renal functions from overall patients. There was a positive correlation between total periostin staining score and serum creatinine ($r = 0.361$, p-value $= 0.010$). On the other hand, there was a negative correlation between total periostin staining score and eGFR ($r = -0.373$, p-value $= 0.008$). These findings were also observed in LN patients (Table 14).

Table 12 Correlation between total periostin staining score and renal pathology

Renal pathology	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Activity index						
Glomerular cell proliferation	-0.018	0.902	0.052	0.762	-0.070	0.820
Fibrinoid necrosis or karyorrhexis	-0.259	0.070	-0.270	0.106	-	-
Cellular crescents	0.196	0.172	0.091	0.591	0.367	0.217
Hyaline thrombi or wire loop	0.220	0.124	0.237	0.158	0.297	0.325
Glomerular leukocyte infiltration	0.138	0.339	0.082	0.631	0.401	0.174
Interstitial inflammation	-0.174	0.228	-0.186	0.269	-0.314	0.296
Total activity index score	0.182	0.206	0.174	0.303	0.224	0.462
Chronicity index						
Glomerular sclerosis	0.417	**0.003***	0.427	**0.008***	0.234	0.442
Segmental sclerosis	0.361	**0.010***	0.280	0.093	0.346	0.247
Global sclerosis	0.171	0.235	0.206	0.221	-0.065	0.832
Fibrous crescent	0.339	**0.016***	0.166	0.325	0.700	**0.008***
Interstitial fibrosis	0.416	**0.003***	0.504	**0.001***	-0.125	0.684
Tubular atrophy	0.416	**0.003***	0.504	**0.001***	-0.125	0.684
Total chronicity index score	0.527	**<0.001***	0.556	**<0.001***	0.255	0.400

*p-value < 0.05

Table 13 Correlation between periostin staining score in each histopathological features of chronicity index

Chronicity index	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Segmental sclerosis	0.719	**<0.001***	0.759	**<0.001***	0.606	**0.028***
Global sclerosis	0.495	**<0.001***	0.518	**0.001***	0.306	0.309
Fibrous crescent	0.526	**<0.001***	-	-	0.850	**<0.001***
Interstitial fibrosis	0.323	**0.022***	0.578	**<0.001***	-0.503	0.080
Tubular atrophy	0.319	**0.024***	0.384	**0.019***	0.124	0.687

*p-value < 0.05

Table 14 Correlation between periostin staining score and other variables

Variables	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Age (year)	0.262	0.066	0.199	0.239	0.332	0.268
Bodyweight (kg)	0.068	0.641	0.014	0.932	-0.069	0.823
Height (cm)	-0.146	0.311	-0.196	0.245	-0.296	0.325
Body mass index (kg/m^2)	0.123	0.395	0.072	0.673	-0.107	0.727
Systolic blood pressure (mmHg)	0.035	0.812	0.069	0.683	-0.047	0.879
Diastolic blood pressure (mmHg)	0.014	0.923	0.078	0.646	-0.007	0.982

Table 14 Correlation between periostin staining score and other variables (cont.)

Variables	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Renal parameters						
Serum creatinine (mg/dl)	0.361	**0.010***	0.377	**0.022***	0.039	0.900
Blood urea nitrogen (mg/dl)	0.198	0.167	0.278	0.096	-0.085	0.782
Serum albumin (g/dl)	-0.134	0.353	-0.345	**0.036***	-0.031	0.921
Urine protein to creatinine ratio	0.185	0.198	0.189	0.263	0.292	0.334
eGFR (ml/min/1.73 m^2) ¶	-0.373	**0.008***	-0.388	**0.018***	-0.006	0.986

¶eGFR: estimated glomerular filtration rate.

*p-value < 0.05

4.2.4 Periostin Staining in Patients with a Low Activity Index Score and a Low Chronicity Index Score

To find out about the advantage of periostin staining over the routine staining, subgroup analysis was further evaluated in patients with a low activity index score (activity index score < 8; n=45) or low chronicity index score (chronicity index score < 4; n=41). Patients were separated into two groups according to median of total periostin staining score from Table 11 as a low periostin staining score (score < 19.3) and a high periostin staining score (score ≥ 19.3). The results found that patients with a high periostin staining score were older than patients with a low periostin staining score in both patients with a low activity index score and a low chronicity index score. A significant higher level of serum creatinine was reported in patients with a high periostin staining score (0.9 (0.7, 1.5) mg/dl) than in those with a low periostin staining score (0.7 (0.6, 0.8) mg/dl) from patients with a low chronicity index score. In contrast, lower eGFR was observed in patients with a high periostin staining score in both patients with a low activity index score and a low chronicity index score. Subgroup analysis in LN patients according to median of total periostin staining score from Table 11 as a low periostin staining score (score < 16.0) and a high periostin staining score (score ≥ 16.0) reported the same. There was a significantly higher serum creatinine and lower eGFR in patients with a high periostin staining score in both patients with a low activity index score and a low chronicity index score. A significantly higher blood urea nitrogen was also observed in patients with a high periostin staining score among patients with a low chronicity index score. Overall results are shown in Table 15-20.

Table 15 Comparison of variables between patients with a high periostin staining score and a low periostin staining score from overall patients with a low activity index score (n=45)

Variables	Patients with a low periostin staining score (n = 23)	Patients with a high periostin staining score (n = 22)	P-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (years)	26.0 (21.0, 38.0) [18.0-46.0]	33.5 (25.0, 50.0) [18.0-59.0]	**0.044***
Body weight (kg)	54.0 (46.0, 64.0) [30.0-85.0]	53.5 (46.0, 64.0) [34.0-85.0]	0.910
Height (cm)	160.0 (153.0, 165.0) [123.0-182.0]	157.5 (151.0, 160.0) [145.0-167.0]	0.180
Body mass index (kg/m^2)	20.1 (19.2, 25.7) [17.5-32.4]	22.5 (18.7, 25.6) [15.5-34.1]	0.708
Systolic blood pressure (mmHg)	131.0 (114.0, 146.0) [93.0-175.0]	129.5 (124.0, 145.0) [108.0-185.0]	0.716
Diastolic blood pressure (mmHg)	81.0 (68.0, 91.0) [56.0-100.0]	83.5 (74.0, 91.0) [46.0-118.0]	0.481
Renal parameters			
Serum creatinine (mg/dl)	0.7 (0.6, 1.1) [0.5-2.7]	0.9 (0.7, 1.1) [0.6-5.2]	0.065
Blood urea nitrogen (mg/dl)	15.6 (11.4, 24.5) [6.3-48.1]	18.8 (12.3, 24.7) [9.2-82.1]	0.376
Serum albumin (g/dl)	3.4 (2.8, 3.8) [1.6-4.3]	3.1 (2.7, 3.9) [2.1-4.3]	0.691
Urine protein to creatinine ratio	1.27 (0.53, 4.28) [0.07-7.93]	2.66 (1.18, 4.48) [0.46-7.99]	0.117
eGFR (ml/min/1.73 m^2) ¶	110.22 (56.80, 128.44) [28.45-170.80]	79.26 (55.66, 94.14) [13.56-128.44]	**0.038***

¶eGFR: estimated glomerular filtration rate. *p-value < 0.05

Table 16 Comparison of variables between patients with a high periostin staining score and a low periostin staining score from LN patients with a low activity index score (n=33)

Variables	Patients with a low periostin staining score (n = 16)	Patients with a high periostin staining score (n = 17)	P-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (years)	26.5 (20.5, 38.0) [18.0-44.0]	25.0 (23.0, 42.0) [20.0-58.0]	0.296
Body weight (kg)	50.5 (45.0, 62.0) [30.0-76.0]	50.0 (46.0, 62.0) [34.0-85.0]	0.759
Height (cm)	157.5 (153.0, 162.5) [123.0-180.0]	158.0 (152.0, 160.0) [145.0-165.0]	0.650
Body mass index (kg/m^2)	19.9 (19.2, 22.6) [17.5-32.4]	21.1 (18.0, 26.0) [15.5-34.1]	0.986
Systolic blood pressure (mmHg)	132.5 (113.0, 149.0) [93.0-175.0]	133.0 (125.0, 145.0) [108.0-185.0]	0.718
Diastolic blood pressure (mmHg)	83.0 (69.5, 95.5) [64.0-100.0]	81.0 (77.0, 91.0) [56.0-112.0]	0.971
Renal parameters			
Serum creatinine (mg/dl)	0.7 (0.6, 0.8) [0.5-1.7]	0.9 (0.7, 0.9) [0.6-1.8]	**0.039***
Blood urea nitrogen (mg/dl)	13.7 (10.7, 23.4) [6.3-31.4]	21.4 (15.4, 28.1) [10.7-60.3]	0.056
Serum albumin (g/dl)	3.4 (2.7, 3.9) [1.8-4.3]	2.9 (2.6, 3.2) [1.6-3.9]	0.165
Urine protein to creatinine ratio	1.27 (0.48, 4.42) [0.07-7.93]	2.74 (1.62, 4.48) [0.46-7.47]	0.150
eGFR (ml/min/1.73 m^2)[¶]	115.59 (85.51, 156.71) [35.75-170.80]	82.47 (68.35, 110.22) [38.13-128.44]	**0.032***

[¶]eGFR: estimated glomerular filtration rate. *p-value < 0.05

Table 17 Comparison of variables between patients with a high periostin staining score and a low periostin staining score from IgAN patients with a low activity index score (n=12)

Variables	Patients with a low periostin staining score (n = 5)	Patients with a high periostin staining score (n = 7)	P-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (years)	26.0 (25.0, 36.0) [21.0-56.0]	50.0 (33.0, 51.0) [18.0-59.0]	0.371
Body weight (kg)	63.0 (58.0, 77.0) [58.0-85.0]	60.0 (49.0, 70.0) [45.0-79.0]	0.328
Height (cm)	165.0 (160.0, 170.0) [151.0-182.0]	160.0 (155.0, 163.0) [150.0, 167.0]	0.220
Body mass index (kg/m^2)	25.4 (24.6, 25.6) [21.3-26.6]	25.0 (19.1, 25.6) [17.6-29.7]	0.465
Systolic blood pressure (mmHg)	131.0 (122.0, 135.0) [120.0-160.0]	125.0 (122.0, 136.0) [109.0-165.0]	0.935
Diastolic blood pressure (mmHg)	77.0 (66.0, 83.0) [46.0-88.0]	85.0 (74.0, 90.0) [65.0-118.0]	0.290
Renal parameters			
Serum creatinine (mg/dl)	1.7 (0.7, 2.3) [0.6-2.7]	1.1 (0.8, 2.3) [0.7-5.2]	0.935
Blood urea nitrogen (mg/dl)	18.1 (13.1, 27.7) [9.2-48.1]	15.6 (12.1, 24.4) [11.5-82.1]	0.935
Serum albumin (g/dl)	3.6 (3.1, 3.8) [2.1-4.3]	4.2 (3.3, 4.3) [2.8-4.3]	0.287
Urine protein to creatinine ratio	1.20 (0.89, 2.30) [0.11-7.99]	1.38 (1.16, 2.56) [0.69-7.42]	0.808
eGFR (ml/min/1.73 m^2) ¶	52.46 (30.51, 92.00) [28.45-120.27]	55.66 (31.08, 87.80] [13.56-94.14]	0.935

¶eGFR: estimated glomerular filtration rate.

Table 18 Comparison of variables between patients with a high periostin staining score and a low periostin staining score from overall patients with a low chronicity index score (n=41)

Variables	Patients with a low periostin staining score (n = 22)	Patients with a high periostin staining score (n = 19)	P-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (years)	25.5 (21.0, 36.0) [18.0-46.0]	31.0 (25.0, 51.0) [21.0-59.0]	**0.030***
Body weight (kg)	53.5 (46.0, 64.0) [30.0-85.0]	56.0 (46.0, 64.0) [34.0-80.0]	0.917
Height (cm)	160.0 (153.0, 165.0) [123.0-182.0]	158.0 (152.0, 160.0) [145.0-178.0]	0.493
Body mass index (kg/m^2)	20.3 (19.2, 25.7) [17.5-32.4]	22.1 (18.7, 25.4) [15.5-33.7]	0.937
Systolic blood pressure (mmHg)	130.5 (114.0, 146.0) [93.0-175.0]	133.0 (124.0, 152.0) [108.0-185.0]	0.346
Diastolic blood pressure (mmHg)	80.5 (71.0, 94.0) [56.0-100.0]	82.0 (68.0, 100.0) [46.0-120.0]	0.565
Renal parameters			
Serum creatinine (mg/dl)	0.7 (0.6, 0.8) [0.5-1.7]	0.9 (0.7, 1.5) [0.6-3.3]	**0.016***
Blood urea nitrogen (mg/dl)	15.5 (11.4, 23.0) [6.3-37.8]	20.7 (12.3, 28.1) [9.2-93.3]	0.170
Serum albumin (g/dl)	3.4 (2.8, 3.8) [1.6-4.3]	3.0 (2.6, 3.8) [2.1-4.3]	0.504
Urine protein to creatinine ratio	1.44 (0.53, 4.28) [0.07-7.93]	2.58 (1.38, 3.91) [0.46-7.99]	0.187
eGFR (ml/min/1.73 m^2) ¶	111.21 (83.21, 128.44) [50.14-170.80]	75.73 (52.44, 108.37) [25.27-128.44]	**0.005***

¶eGFR: estimated glomerular filtration rate. *p-value < 0.05

Table 19 Comparison of variables between patients with a high periostin staining score and a low periostin staining score from LN patients with a low chronicity index score (n=34)

Variables	Patients with a low periostin staining score (n = 17)	Patients with a high periostin staining score (n = 17)	P-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (years)	26.0 (21.0, 33.0) [18.0-44.0]	25.0 (23.0, 35.0) [21.0-58.0]	0.277
Body weight (kg)	51.0 (45.0, 64.0) [30.0-76.0]	51.0 (46.0, 62.0) [34.0-80.0]	0.986
Height (cm)	160.0 (153.0, 165.0) [123.0-180.0]	158.0 (153.0, 160.0) [145.0-178.0]	0.835
Body mass index (kg/m^2)	20.1 (19.2, 23.1) [17.5-32.4]	21.9 (18.0, 24.8) [15.5-33.7]	0.730
Systolic blood pressure (mmHg)	138.0 (114.0, 152.0) [93.0-175.0]	133.0 (125.0, 145.0) [108.0-185.0]	0.692
Diastolic blood pressure (mmHg)	85.0 (71.0, 97.0) [64.0-100.0]	81.0 (77.0, 91.0) [56.0-120.0]	0.972
Renal parameters			
Serum creatinine (mg/dl)	0.7 (0.6, 0.8) [0.5-1.3]	0.9 (0.7, 1.1) [0.6-3.3]	**0.023***
Blood urea nitrogen (mg/dl)	13.8 (11.3, 23.0) [6.3-37.8]	21.4 (15.4, 28.1) [10.7-93.3]	**0.044***
Serum albumin (g/dl)	3.4 (2.8, 3.8) [1.8-4.3]	3.0 (2.6, 3.3) [1.6-3.9]	0.152
Urine protein to creatinine ratio	1.61 (0.53, 4.28) [0.07-7.93]	2.74 (1.62, 3.91) [0.46-7.47]	0.256
eGFR (ml/min/1.73 m^2) ¶	112.27 (87.80, 156.12) [50.14-170.80]	81.76 (64.32, 110.22) [25.27-128.44]	**0.014***

¶eGFR: estimated glomerular filtration rate. *p-value < 0.05

Table 20 Comparison of variables between patients with a high periostin staining score and a low periostin staining score from IgAN patients with a low chronicity index score (n=7)

Variables	Patients with a low periostin staining score (n = 3)	Patients with a high periostin staining score (n = 4)	P-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (years)	36.0 (25.0, 56.0) [25.0-56.0]	51.0 (50.5, 55.0) [50.0-59.0]	0.285
Body weight (kg)	63.0 (58.0, 85.0) [58.0-85.0]	61.5 (54.4, 66.5) [49.0-70.0]	0.593
Height (cm)	160.0 (151.0, 182.0) [151.0, 182.0]	158.5 (156.0, 163.5) [155.0-167.0]	0.858
Body mass index (kg/m^2)	25.4 (24.6, 25.7) [24.6-25.7]	25.0 (22.1, 25.3) [19.1-25.6]	0.480
Systolic blood pressure (mmHg)	122.0 (120.0, 135.0) [120.0-135.0]	130.0 (123.0, 150.5) [122.0-165.0]	0.212
Diastolic blood pressure (mmHg)	77.0 (46.0, 88.0) [46.0-88.0]	85.0 (72.5, 104.0) [65.0-118.0]	0.289
Renal parameters			
Serum creatinine (mg/dl)	0.7 (0.6, 1.7) [0.6-1.7]	1.3 (0.9, 1.9) [0.7-2.3]	0.372
Blood urea nitrogen (mg/dl)	13.1 (9.2, 18.1) [9.2-18.1]	17.4 (12.8, 22.6) [11.5-24.4]	0.289
Serum albumin (g/dl)	3.1 (2.1, 4.3) [2.1-4.3]	4.2 (3.5, 4.3) [2.8-4.3]	0.589
Urine protein to creatinine ratio	1.20 (0.11, 7.99) [0.11-7.99]	1.58 (1.27, 2.17) [1.16-2.56]	0.724
eGFR ($ml/min/1.73\ m^2$) ¶	92.00 (52.46, 120.27) [52.46-120.27]	54.05 (41.76, 74.90) [31.08-94.14]	0.289

¶eGFR: estimated glomerular filtration rate.

4.3 Periostin mRNA Expression Evaluation

There were five control kidney tissues and 13 kidney tissues from patients that could be obtained for mRNA expression analysis. The results showed that periostin mRNA expression was not significantly different between patient and control kidney tissues. In contrast, significantly higher level of TGF-β mRNA expression was observed in patients (4.23 (2.55, 12.08) fold change) than in controls (0.94 (0.87, 1.63) fold change). Overall mRNA expression analysis is shown in Table 21.

Table 21 The mRNA expression of target genes in kidney tissues from controls and patients

Target genes	mRNA expression (fold change) Median (Q1, Q3) [Range]		p-value
	Control (n=5)	Patients (n=13)	
Periostin	1.05 (0.75, 1.46) [0.47-1.88]	1.21 (0.77, 1.71) [0.09-4.07]	0.730
TGF-β	0.94 (0.87, 1.63) [0.34-2.23]	4.23 (2.55, 12.08) [1.05-15.87]	**0.003***

*p-value < 0.05

4.4 Urine Periostin Evaluation

4.4.1 Urine Periostin Level Measurement

Urine periostin level was measured in 50 patients and 50 healthy controls. There was a significantly higher level of urine periostin in patients than in healthy controls from overall patient and subgroup analysis in LN patients and IgA nephropathy patients (Table 22). The results showed that urine periostin levels were detected in 23 out of 50 patients and 11 out of 50 healthy controls. The result of urine periostin level in patients

and healthy controls with urine periostin detection is shown in Table 23. The median value of urine periostin levels from patients (33.27 (9.89, 158.60) ng/mg) was statistically significantly higher than in controls (2.38 (1.34, 6.54) ng/mg); ($p < 0.001$). Moreover, the same results were also reported in subgroup analysis from lupus nephritis patients and IgA nephropathy patients. Urine periostin level was detected in 17 lupus nephritis patients and 6 IgA nephropathy patients with median values of 33.27 (11.74, 124.44) ng/mg and 27.23 (9.89, 159.32) ng/mg, respectively. A statistically significant difference of urine periostin level between lupus nephritis patients and controls, as well as between IgA nephropathy patients and controls, was also reported ($p< 0.05$).

Table 22 Urine periostin level in overall patients and healthy controls

Subjects	Urine periostin level (ng/mg**)		p-value
	Median (Q1, Q3) [Range]	Mean ± SE	
Overall patients (n=50)	0 (0, 22.61) [0-570.87]	45.16 ± 15.14	**0.001***
LN patients (n=37)	0 (0, 22.61) [0-570.87]	45.30 ± 18.10	**0.002***
IgAN patients (n=13)	0 (0, 18.76) [0-351.78]	44.77 ± 28.29	**0.021***
Healthy controls (n=50)	0 (0, 0) [0-19.69]	1.02 ± 0.45	-

*p-value<0.05 (comparison with healthy controls)

** mg of urine creatinine

Table 23 Urine periostin level in patients and healthy controls with urine periostin detection

Subjects with urine periostin detection	Urine periostin level (ng/mg**) Median (Q1, Q3) [Range]	p-value
Patients (n=23)	33.27 (9.89, 158.60) [2.24-570.87]	< 0.001*
LN patients (n=17)	33.27 (11.74, 124.44) [2.24-570.87]	< 0.001*
IgAN patients (n=6)	27.23 (9.89, 159.32) [6.53-351.78]	0.005*
Healthy controls (n=11)	2.38 (1.34, 6.54) [0.28-19.69]	-

*p-value<0.05 (comparison with healthy controls)

** mg of urine creatinine

From the previous results, a subgroup analysis between patients with urine periostin detection and without urine periostin detection was performed (Table 24). There was a statistically significant difference between serum creatinine in patients with urine periostin detection (0.9 (0.7, 1.8) mg/dl) and patients without urine periostin detection (0.7 (0.6, 1.0) mg/dl). In addition, the eGFR tendency was lower in patients with urine periostin detection than in those without urine periostin detection.

Table 24 Comparison of variables between patients with urine periostin detection and patients without urine periostin detection

Variables	Patients with urine periostin detection (n = 23)	Patients without urine periostin detection (n = 27)	P-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (years)	26.0 (21.0, 34.0) [18.0-59.0]	30.0 (22.0, 42.0) [18.0-58.0]	0.360
Body weight (kg)	60.0 (49.0, 70.0) [37.0-79.0]	51.0 (46.0, 63.0) [30.0-85.0]	0.255
Height (cm)	160.0 (156.0, 165.0) [136.0-178.0]	158.0 (152.0, 165.0) [123.0-182.0]	0.206
Body mass index (kg/m^2)	22.2 (19.5, 26.1) [16.4-32.4]	21.1 (19.1, 24.6) [15.5-34.1]	0.271
Systolic blood pressure (mmHg)	133.0 (124.0, 155.0) [93.0-170.0]	128.0 (115.0, 150.0) [104.0-185.0]	0.430
Diastolic blood pressure (mmHg)	81.0 (77.0, 97.0) [60.0-120.0]	82.0 (71.0, 91.0) [46.0-112.0]	0.915
Renal parameters			
Serum creatinine (mg/dl)	0.9 (0.7, 1.8) [0.5-5.2]	0.7 (0.6, 1.0) [0.5-1.8]	**0.023***
Blood urea nitrogen (mg/dl)	23.0 (13.8, 37.8) [10.1-93.3]	16.8 (12.3, 23.7) [6.3-56.9]	0.094
Serum albumin (g/dl)	3.1 (2.7, 3.7) [1.6-4.3]	3.4 (2.8, 3.9) [1.8-4.3]	0.306
Urine protein to creatinine ratio	2.56 (1.38, 4.48) [0.34-7.47]	1.61 (0.69, 4.55) [0.07-7.99]	0.271
eGFR (ml/min/1.73 m^2) ¶	74.79 (41.03, 120.81) [13.32-141.75]	97.18 (70.71, 123.38) [37.72-138.79]	0.059

¶eGFR: estimated glomerular filtration rate

*p-value < 0.05

4.4.2 Correlation of Urine Periostin Level

The correlation between urine periostin level and renal pathology was also assessed (Table 25-26). No correlation was found between urine periostin level and renal pathology including activity index score, chronicity index score and total periostin staining score. The same results were also observed for each renal pathology within activity index, chronicity index and periostin staining analysis. However, there was a tendency of correlation between urine periostin level and interstitial fibrosis ($r = 0.547$; p-value = 0.053) as well as tubular atrophy ($r = 0.547$; p-value = 0.053) in patients with IgA nephropathy. On the other hand, there was a significant correlation between urine periostin level and renal functions (Table 27). In LN patients, a positive correlation was reported between urine periostin level and serum creatinine ($r = 0.399$; p-value = 0.014). In IgA nephropathy patients, urine periostin level was also correlated with serum creatinine ($r = 0.639$; p-value = 0.019) as well as blood urea nitrogen ($r = 0.615$; p-value = 0.025). There was also a negative correlation between urine periostin level and eGFR ($r = -0.687$; p-value = 0.009).

Table 25 Correlation between urine periostin level and renal pathology

Renal pathology	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Activity index						
Glomerular cell proliferation	-0.094	0.518	0.027	0.875	-0.438	0.135
Fibrinoid necrosis or karyorrhexis	0.216	0.132	0.256	0.126	-	-
Cellular crescents	0.234	0.102	0.253	0.131	0.161	0.598
Hyaline thrombi or wire loop	0.128	0.374	0.132	0.435	0.121	0.694
Glomerular leukocyte infiltration	0.139	0.335	0.276	0.098	-0.291	0.336
Interstitial inflammation	0.143	0.320	0.147	0.387	0.124	0.687
Total activity index score	0.173	0.229	0.265	0.113	-0.030	0.922
Chronicity index						
Glomerular sclerosis	-0.094	0.518	-0.211	0.210	0.298	0.323
Segmental sclerosis	-0.104	0.472	-0.253	0.131	0.268	0.377
Global sclerosis	0.077	0.594	0.004	0.983	0.304	0.312
Fibrous crescent	0.017	0.905	0.080	0.637	-0.077	0.803
Interstitial fibrosis	0.123	0.396	0.037	0.829	0.547	0.053
Tubular atrophy	0.123	0.396	0.037	0.829	0.547	0.053
Total chronicity index score	0.022	0.879	-0.064	0.707	0.413	0.161

Table 26 Correlation between urine periostin level and periostin staining score

Periostin staining	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Periglomerular staining	0.191	0.185	0.175	0.300	0.255	0.400
Mesangial staining	0.109	0.450	0.195	0.248	-0.124	0.687
Fibrocellular crescent staining	0.041	0.776	0.065	0.702	-0.036	0.906
Fibrous crescent staining	0.012	0.934	-	-	0.073	0.813
Segmental sclerosis staining	-0.209	0.146	-0.250	0.135	-0.174	0.570
Global sclerosis staining	-0.198	0.167	-0.274	0.101	-0.114	0.710
Interstitial fibrosis staining	0.172	0.232	0.217	0.196	-0.028	0.928
Vascular fibrosis staining	0.056	0.700	0.078	0.646	-0.054	0.861
Tubular epithelial cell staining	0.071	0.626	0.014	0.932	0.280	0.353
Tubular atrophy staining	0.057	0.697	-0.032	0.852	0.304	0.312
Tubular cell cast staining	-0.074	0.612	-0.070	0.681	0.003	0.992
Total periostin staining score	0.084	0.562	0.073	0.667	0.120	0.697

Table 27 Correlation between urine periostin level and other variables

Variables	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Age (year)	-0.130	0.369	-0.201	0.234	0.078	0.801
Bodyweight (kg)	0.222	0.121	0.153	0.365	0.434	0.138
Height (cm)	0.221	0.123	0.234	0.163	0.126	0.681
Body mass index (kg/m^2)	0.221	0.123	0.141	0.405	0.573	**0.040***
Systolic blood pressure (mmHg)	0.118	0.415	0.116	0.496	0.135	0.661
Diastolic blood pressure (mmHg)	0.005	0.973	0.009	0.960	-0.063	0.838
Renal parameters						
Serum creatinine (mg/dl)	0.410	**0.003***	0.399	**0.014***	0.639	**0.019***
Blood urea nitrogen (mg/dl)	0.355	**0.011***	0.265	0.112	0.615	**0.025***
Serum albumin (g/dl)	-0.148	0.304	-0.186	0.270	-0.289	0.338
Urine protein to creatinine ratio	0.218	0.129	0.140	0.408	0.478	0.099
eGFR ($ml/min/1.73\ m^2$) ¶	-0.399	**0.004***	-0.320	0.054	-0.687	**0.009***

¶eGFR: estimated glomerular filtration rate.

*p-value < 0.05

4.4.3 Receiver Operating Characteristics Analysis of Urine Periostin Level

Receiver operating characteristic curves of the urine periostin level were generated to find out the best cutoff value of the urine periostin level. The area under the curve for urine periostin level from overall patients, LN patients and IgA nephropathy patients were 0.661 (95% CI, 0.553-0.770), 0.661 (95% CI, 0.539-0.782) and 0.664 (95% CI, 0.471-0.857), respectively. The best urine periostin level cutoff value of LN patients was 2.098 ng/mg, with a sensitivity and specificity of 45.9% and 88.0%, respectively. Positive predictive value, negative predictive value and accuracy were 73.9%, 68.8% and 70.1%. In IgA nephropathy patients, the best urine periostin level cutoff value was 5.775 ng/mg, with a sensitivity and specificity of 46.2% and 94.0%, respectively. Positive predictive value, negative predictive value and accuracy were 66.7%, 87.0% and 84.1%. Overall results are shown in Table 28.

Table 28 Diagnostic tests of the urine periostin level

Patients	Cutoff (ng/mg)	Sensitivity [95%CI]	Specificity [95%CI]	PPV [95%CI]	NPV [95%CI]	Accuracy [95%CI]
Overall patients (n=50)	≥ 2.098	46.0% [28.4-63.6]	88.0% [76.5-99.5]	79.3% [60.5-98.1]	62.0% [47.6-76.4]	67.0% [55.2-78.8]
LN patients (n=37)	≥ 2.098	45.9% [25.5-66.4]	88.0% [76.5-99.5]	73.9% [51-96.8]	68.8% [54.3-83.2]	70.1% [57.8-82.4]
IgAN patients (n=13)	≥ 5.775	46.2% [11.6-80.7]	94.0% [85.6-100]	66.7% [27.4-100]	87.0% [75.6-98.5]	84.1% [72.6-95.6]

PPV: positive predictive value; NPV: negative predictive value; CI: confidence interval

4.5 Serum Periostin Evaluation

4.5.1 Serum Periostin Level Measurement

The results showed that the median serum periostin level in healthy controls was 631.07 (113.28, 8570.98) ng/ml. The median serum periostin level in overall patient was 439.59 (196.04, 1260.84) ng/ml. There was no statistically significant difference between the serum periostin level in patients and healthy controls ($p>0.05$). Subgroup analysis in LN and IgA nephropathy patients reported the same. Overall results are shown in Table 29.

Table 29 Serum periostin level in patients and healthy controls

Subjects	Serum periostin level (ng/ml) Median (Q1, Q3) [Range]	p-value
Overall patients (n=50)	439.59 (196.04, 1260.84) [58.03-18312.62]	0.730
LN patients (n=37)	454.78 (166.92, 2908.43) [58.03-18312.62]	0.687
IgAN patients (n=13)	424.39 (291.44, 668.82) [118.64-5692.43]	0.959
Healthy controls (n=50)	631.07 (113.28, 8570.98) [40.28-24687.94]	-

4.5.2 Correlation of Serum Periostin Level

There was a correlation between serum periostin level and renal pathology in some histologic features (Table 30). The results from overall patients and LN patients showed that there was a correlation between serum periostin level and segmental sclerosis. In addition, serum periostin level was correlated with glomerular cell proliferation in IgA nephropathy patients ($r=0.602$; p-value = 0.030). The correlation between serum periostin level and periostin staining score was also demonstrated (Table 31). No correlation was found between serum periostin level and periostin staining score from subgroup analysis in LN and IgA nephropathy patients. Additionally, there was a correlation between serum periostin level and body weight from overall patients and LN patients (Table 32). In contrast, serum periostin level correlated with diastolic blood pressure and serum albumin in IgA nephropathy patients. No correlation was found between serum periostin level and urine periostin level from overall patient and subgroup analysis in LN and IgA nephropathy patients.

Table 30 Correlation between serum periostin level and renal pathology

Renal pathology	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Activity index						
Glomerular cell proliferation	0.207	0.150	0.115	0.500	0.602	**0.030***
Fibrinoid necrosis or karyorrhexis	-0.269	0.059	-0.291	0.080	-	-
Cellular crescents	-0.207	0.149	-0.207	0.219	-0.212	0.487
Hyaline thrombi or wire loop	-0.081	0.576	-0.006	0.970	-0.489	0.090
Glomerular leukocyte infiltration	-0.225	0.115	-0.252	0.133	-0.156	0.611
Interstitial inflammation	-0.196	0.172	-0.297	0.074	0.057	0.853
Total activity index score	-0.067	0.642	-0.086	0.612	-0.028	0.928
Chronicity index						
Glomerular sclerosis	0.081	0.576	0.128	0.450	-0.130	0.672
Segmental sclerosis	0.291	**0.040***	0.357	**0.030***	0.053	0.864
Global sclerosis	-0.168	0.242	-0.171	0.311	-0.174	0.571
Fibrous crescent	0.062	0.669	-0.035	0.836	0.340	0.256
Interstitial fibrosis	-0.043	0.768	-0.073	0.668	-0.160	0.602
Tubular atrophy	-0.043	0.768	-0.073	0.668	-0.160	0.602
Total chronicity index score	0.019	0.894	0.009	0.956	-0.104	0.735

*p-value < 0.05

Table 31 Correlation between serum periostin level and periostin staining score

Periostin staining	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Periglomerular staining	0.327	**0.020***	0.319	0.054	0.312	0.300
Mesangial staining	0.123	0.394	0.073	0.666	0.342	0.252
Fibrocellular crescent staining	0.096	0.508	0.082	0.628	0.168	0.584
Fibrous crescent staining	0.118	0.415	-	-	0.268	0.376
Segmental sclerosis staining	0.254	0.075	0.311	0.061	0.116	0.705
Global sclerosis staining	0.104	0.474	0.024	0.886	0.305	0.311
Interstitial fibrosis staining	0.077	0.597	0.028	0.871	0.160	0.602
Vascular fibrosis staining	-0.023	0.874	-0.061	0.722	0.064	0.835
Tubular epithelial cell staining	0.019	0.895	0.088	0.603	-0.238	0.435
Tubular atrophy staining	0.128	0.376	0.147	0.385	0.078	0.799
Tubular cell cast staining	0.194	0.176	0.194	0.250	0.181	0.554
Total periostin staining score	0.215	0.134	0.216	0.200	0.267	0.377

*p-value < 0.05

Table 32 Correlation between serum periostin level and other variables

Variables	Overall Patients (n=50)		LN Patients (n=37)		IgAN Patients (n=13)	
	Correlation	p-value	Correlation	p-value	Correlation	p-value
Age (year)	-0.197	0.170	-0.274	0.101	0.000	1.000
Bodyweight (kg)	-0.292	**0.040***	-0.340	**0.040***	-0.223	0.463
Height (cm)	-0.220	0.125	-0.277	0.097	-0.083	0.787
Body mass index (kg/m^2)	-0.248	0.083	-0.265	0.113	-0.253	0.404
Systolic blood pressure (mmHg)	-0.167	0.247	-0.183	0.278	0.057	0.854
Diastolic blood pressure (mmHg)	-0.012	0.936	-0.115	0.496	0.603	**0.029***
Renal parameters						
Serum creatinine (mg/dl)	-0.003	0.986	-0.042	0.805	-0.037	0.904
Blood urea nitrogen (mg/dl)	-0.020	0.892	0.005	0.978	-0.074	0.809
Serum albumin (g/dl)	0.144	0.318	0.002	0.990	0.730	**0.005***
Urine protein to creatinine ratio	-0.067	0.642	-0.045	0.790	-0.226	0.459
eGFR ($ml/min/1.73\ m^2$) ¶	0.044	0.764	0.119	0.481	0.008	0.979
Urine periostin level (ng/mg)	-0.175	0.223	-0.126	0.457	-0.413	0.161

¶eGFR: estimated glomerular filtration rate

*p-value < 0.05

4.6 Prediction of Clinical Response to Therapy after 6 months of Treatment

Thirty-six out of 50 patients could be followed up after 6 months of treatment. Twenty patients were classified as patients with response to therapy and 16 patients as non-response to therapy. Renal pathology, periostin staining and characteristic data at baseline were compared between patients with response and non-response to therapy (Table 33-35). The results found that the activity index score, chronicity index score and total periostin staining score were not different between patients with response and non-response to therapy. Other variables including characteristic data, renal parameters, treatment, urine periostin level and serum periostin level were not different between patients with response and non-response to therapy.

Table 33 Comparison of renal pathology and clinical response after 6 months of treatment

Renal pathology	Patients with response to therapy (n=20)	Patients with non-response to therapy (n=16)	p-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Activity index			
Glomerular cell proliferation	2 (1, 3) [0-3]	3 (1, 3) [0-3]	0.686
Fibrinoid necrosis or karyorrhexis	0 (0, 0) [0-2]	0 (0, 0) [0-2]	0.199
Cellular crescents	0 (0, 0) [0-4]	0 (0, 0) [0-2]	0.101
Hyaline thrombi or wire loop	1 (0, 2) [0-3]	0 (0, 1) [0-3]	0.226
Glomerular leukocyte infiltration	0 (0, 1) [0-2]	0 (0, 0) [0-2]	0.231

Table 33 Comparison of renal pathology and clinical response after 6 months of treatment (cont.)

Renal pathology	Patients with response to therapy (n=20)	Patients with non-response to therapy (n=16)	p-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Interstitial inflammation	0 (0, 0) [0-1]	0 (0, 0) [0-1]	0.413
Total activity index score	4 (2, 8) [1-11]	3 (2, 5) [0-9]	0.163
Chronicity index			
Glomerular sclerosis	0 (0, 1) [0-2]	0 (0, 1) [0-2]	0.871
Segmental sclerosis	0 (0, 1) [0-2]	0 (0, 1) [0-2]	0.612
Global sclerosis	0 (0, 1) [0-1]	0 (0, 0) [0-2]	0.358
Fibrous crescent	0 (0, 1) [0-3]	0 (0, 0) [0-1]	0.072
Interstitial fibrosis	1 (0, 1) [0-2]	1 (1, 2) [0-3]	0.064
Tubular atrophy	1 (0, 1) [0-2]	1 (1, 2) [0-3]	0.064
Total chronicity index score	3 (0, 4) [0-7]	2 (2, 4) [0-8]	0.603

Table 34 Comparison of periostin staining score and clinical response after 6 months of treatment

Periostin staining	Patients with response to therapy (n=20)	Patients with non-response to therapy (n=16)	p-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Periglomerular staining	0 (0, 7) [0-12]	3 (0, 9) [0-15]	0.363
Mesangial staining	0 (0, 0) [0-0]	0 (0, 0) [0-5]	**0.046***
Fibrocellular crescent staining	0 (0, 1) [0-9]	0 (0, 0) [0-4]	0.128
Fibrous crescent staining	0 (0, 0) [0-8]	0 (0, 0) [0-6]	0.904
Segmental sclerosis staining	0 (0, 0) [0-6]	0 (0, 2) [0-6]	0.489
Global sclerosis staining	0 (0, 1) [0-4]	0 (0, 0) [0-3]	0.381
Interstitial fibrosis staining	0 (0, 3) [0-6]	1 (0, 4) [0-6]	0.630
Vascular fibrosis staining	0 (0, 0) [0-10]	0 (0, 1) [0-15]	0.776
Tubular epithelial cell staining	1 (0, 6) [0-10]	2 (0, 4) [0-6]	0.604
Tubular atrophy staining	0 (0, 2) [0-6]	0 (0, 1) [0-3]	0.572
Tubular cell cast staining	1 (0, 5.5) [0-10]	0 (0, 2.5) [0-9]	0.369
Total periostin staining score	5.5 (0, 29.5) [0-65]	11.5 (2.5, 27.5) [0-36]	0.689

*p-value < 0.05

Table 35 Comparison of variables and clinical response after 6 months of treatment

Variables	Patients with response to therapy (n=20)	Patients with non-response to therapy (n=16)	p-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Age (year)	27.5 (22.0, 39.5) [18.0-59.0]	24.5 (21.0, 36.5) [18.0-51.0]	0.398
Bodyweight (kg)	59.5 (48.0, 64.0) [30.0-85.0]	52.5 (46.0, 71.5) [40.0-85.0]	0.899
Height (cm)	159.0 (153.0, 165.0) [123.0-180.0]	160.0 (154.0, 167.0) [150.0-182.0]	0.422
Body mass index (kg/m^2)	22.1 (20.0, 24.9) [17.5-34.1]	20.7 (18.6, 26.2) [16.4-33.7]	0.426
Systolic blood pressure (mmHg)	145.0 (124.5, 153.0) [104.0-175.0]	129.5 (122.0, 140.0) [93.0-170.0]	0.390
Diastolic blood pressure (mmHg)	86.0 (73.0, 99.5) [60.0-120.0]	83.5 (78.5, 89.5) [64.0-118.0]	0.786
Renal parameters			
Serum creatinine (mg/dl)	0.7 (0.6, 1.0) [0.5-2.3]	0.9 (0.8, 1.6) [0.5-3.3]	0.052
Blood urea nitrogen (mg/dl)	16.3 (12.2, 24.1) [6.3-52.2]	16.1 (12.6, 24.2) [10.1-93.3]	0.937
Serum albumin (g/dl)	3.2 (2.8, 3.5) [1.8-4.2]	3.7 (3.0, 4.1) [2.4-4.3]	0.077
Urine protein to creatinine ratio	2.44 (1.04, 5.68) [0.07-7.93]	1.29 (0.73, 2.44) [0.34-4.55]	0.157
eGFR (ml/min/1.73 m^2)¶	109.79 (67.38, 123.40) [31.08-165.54]	76.24 (52.45, 99.01) [25.27-170.80]	0.072

Table 35 Comparison of variables and clinical response after 6 months of treatment (cont.)

Variables	Patients with response to therapy (n=20)	Patients with non-response to therapy (n=16)	p-value
	Median (Q1, Q3) [Range]	Median (Q1, Q3) [Range]	
Treatment (n, %)			
Prednisolone	20 (100%)	14 (88%)	0.190
Cyclophosphamide	11 (55%)	5 (31%)	0.154
Mycophenolate mofetil	6 (30%)	5 (31%)	0.936
Azathioprine	4 (20%)	3 (19%)	1.000
ACEI / ARB	12 (60%)	10 (63%)	0.878
Urine periostin level (ng/mg)	0 (0, 34.73) [0-254.31]	4.49 (0, 17.17) [0-159.32]	0.863
Serum periostin level (ng/ml)	327.39 (163.46, 551.40) [85.23-7892.52]	570.05 (166.43, 4409.44) [58.03-11603.28]	0.324

¶eGFR: estimated glomerular filtration rate; ACEI: angiotensin converting enzyme inhibitors; ARB: angiotensin receptor blockers

After 6 months of treatment, urine samples were collected from patients with urine periostin detection on the biopsy date for urine periostin measurement. Sixteen out of 23 patients with urine periostin detection on the biopsy date could be followed up after 6 months of treatment. Among these patients, seven were classified as patients with response to therapy and nine were classified as patients with non-response to therapy. The results showed that there was a significantly decreased urine periostin level after 6 months of treatment in patients with response to therapy as shown in Table 36.

Table 36 Urine periostin level in patients with response and non-response to therapy at biopsy date and after 6 months of treatment

Response to therapy	Urine periostin level (ng/mg) Median (Q1, Q3) [Range]		p-value
	At biopsy date	After 6 months of treatment	
Patients with response to therapy (n=7)	35.70 (33.27, 158.60) [12.68-252.83]	4.35 (0.47, 21.17) [0.10-24.01]	**0.028***
Patients with non-response to therapy (n=9)	11.74 (6.53, 69.96) [2.47-159.32]	7.45 (5.64, 20.66) [0.02-35.16]	0.173
p-value	0.091	0.536	

*p-value < 0.05

CHAPTER V

DISCUSSION

Periostin is a novel biomarker that has been studied in both animals and humans with different types of kidney diseases. In animal studies, no periostin staining was observed from control kidney tissues. In contrast, positive periostin staining was detected in kidney tissues from animals with various types of kidney injury including ureteral obstruction, diabetic nephropathy and hypertensive nephropathy [18, 19]. In human studies, periostin was not detected in control kidney tissues. Positive periostin staining was found in kidney tissue samples from patients with chronic allograft nephropathy and diabetic nephropathy [95, 96]. The same tendency was reported in our study. Periostin staining was not detected in control kidney tissues. In contrast, periostin staining was observed in glomeruli, the interstitial area, tubules and renal vessels in kidney samples from patients with lupus nephritis and IgA nephropathy. According to periostin staining analysis, tubular epithelial cell staining was the most common finding in our study. In addition, tubular cell casts and tubular atrophy staining were observed in 52% and 40% of overall patients, respectively. In previous study, positive periostin staining was found in the tubular epithelial cells, tubular casts or sloughed cells within the tubular lumen from animal models with nephrectomy, ureteral obstruction and diabetic nephropathy [18]. In patients with diabetic nephropathy, periostin was also detected in both non-atrophic and atrophic tubular epithelial cells [96]. In addition, positive periostin was found in tubular epithelial cells and tubular atrophy from patients with chronic allograft nephropathy [19, 95]. In our study, periglomerular staining was also found in more than half of the patients. Moreover, areas with fibrosis including glomeruli, interstitium and vessels were also positive for periostin. According to previous study, serial kidney tissue sections from diabetic nephropathy patients also showed a

positive periostin area in nodular glomerulosclerosis, periglomerular fibrosis, mesangial areas and interstitial fibrosis [96]. The same results were reported in patients with chronic allograft nephropathy including areas with glomerular fibrosis, periglomeruli and interstitial fibrosis [19, 95]. In our study, the correlation between total periostin staining score and renal pathology including activity index score and chronicity index score was evaluated. There was no correlation between total periostin staining score and activity index score. However, a positive correlation was found between total periostin staining score and chronicity index score. These results suggest that periostin related to chronic of kidney diseases. Moreover, periostin was also significantly correlated with some pathological features that represented the chronicity of disease including segmental sclerosis, fibrous crescent, interstitial fibrosis and tubular atrophy. Supporting these results, there was more diffusion and greater intensity of periostin staining in kidney tissues over time after chronic kidney injury in animals with nephrectomy, diabetic nephropathy and ureteral obstruction [18]. Positive periostin areas were found in obsolescent glomeruli, interstitial cells, tubular cells and renal vessels with more intensity over time after kidney injury. Moreover, periostin mRNA expression within kidney tissues also increased over time with statistically significant difference compared with control kidney tissue at each time point [18, 19]. In patients with various proteinuric kidney diseases, periostin mRNA was the strongest induction compared with other matricellular proteins. A significantly higher level of periostin mRNA expression from glomeruli was found in patients with progressive glomerulonephropathies including lupus nephritis and focal segmental glomerulosclerosis. A tendency for periostin mRNA induction was observed in IgA nephropathy. However, there was no statistically significant difference compared with control kidney tissue, which may be due to the variance of periostin steady-state expression [20]. In our study, periostin mRNA expression was also performed using real-time PCR. However, the results showed that there was no statistically significant difference between patients and control kidney tissues.

Renal fibrosis is a common pathway leading to end-stage renal disease, regardless of etiology. The final pathological features presented within kidney tissue are the same including glomerular sclerosis, interstitial fibrosis and tubular atrophy. EMT is considered to be a major process involved in the progression of kidney disease [8]. It is a stepwise process mainly induced by TGF-β. The process is initiated by loss of tubular epithelial cell-cell adhesion. The transition of tubular epithelial cells to myofibroblasts was observed to be represented by the disappearance of tubular epithelial markers together with *de novo* expression of mesenchymal markers. Tubular basement membrane disruption leads to the migration and invasion of myofibroblasts into the interstitial area [98]. The accumulation of extracellular matrix protein produced by myofibroblasts leads to the fibrosis and deterioration of kidney function. In our study, there was a statistical increase in TGF-β mRNA expression in patients compared with controls. In previous studies, TGF-β expression was also observed in both animals and humans with kidney diseases. There was a strong induction of TGF-β at 15 days from animals with unilateral ureteral obstruction [22]. In patients with chronic glomerulonephritis, TGF-β immunolabeling was detected in glomeruli and the interstitial areas. Moreover, there was a significant positive correlation between interstitial TGF-β immunolabeling and areas with interstitial fibrosis, vascular hyalinosis/fibrosis and tubular atrophy as well as a total chronicity index [128]. From our results, we found that periostin was observed in both glomeruli and tubulointerstitial areas, especially in areas with fibrosis. There was also a significant correlation between total periostin staining score and chronicity index score as well as some pathological characteristics of chronic kidney disease progression including segmental sclerosis, fibrous crescent, interstitial fibrosis and tubular atrophy. We supposed that the periostin expression within kidney tissue may be activated by TGF-β after chronic kidney injury. Supporting this hypothesis, there was a dose-dependent periostin mRNA expression after being induced by TGF-β. In murine mesangial cells, there was a significantly higher of periostin mRNA expression after incubation with TGF-β for 4 hours at concentrations of 1 ng/ml and 10 ng/ml compared with vehicle controls. In addition, mesangial cells were

considered to be the source of periostin expression in glomeruli [20]. In human collecting duct cells, an increase in periostin mRNA expression was also observed in a dose-dependent pattern after exposed to TGF-β. In contrast, there was no effect of TGF-β after being induced by periostin [22]. These results confirmed that TGF-β was a stimulator of periostin. Moreover, a significant positive correlation between TGF-β and periostin mRNA expression was also reported in both glomeruli and tubulointerstitium from patients with different nephropathies [20]. The relevance of periostin and EMT markers was also found in both animal and human studies. Immunofluorescence analysis in remnant kidney rat samples after nephrectomy showed that there was a disappearance of E-cadherin, an epithelial marker, in the distal tubules expressing periostin. In addition, serial sections of immunohistochemistry staining revealed a co-localization of EMT markers including fibroblast-specific protein 1 and matrix metalloproteinase-9 together with periostin in tubular epithelial cells, fragments of tubular cells in the lumen and interstitial cells at all times after kidney injury [18]. To confirm the effect of periostin on EMT markers, *in vitro* transfection of periostin cDNA into mouse distal collecting tubular (MDCT) cells was investigated. There was the obvious increase of fibroblast-specific protein 1 and matrix metalloproteinase-9 together with a decrease of E-cadherin expression in periostin-overexpressed MDCT cells. In contrast, MDCT cells co-transfected with periostin cDNA and knockdown periostin gene with SureSilencing short interfering RNA showed a marked reduction of periostin protein level together with the reverse effect on fibroblast-specific protein 1, matrix metalloproteinase-9 and E-cadherin. These results demonstrated that the expression of EMT markers in tubular cells was induced by periostin [18]. In addition, an increase in mesenchymal marker vimentin mRNA expression was observed in animals with hypertensive nephropathy [19]. The association between periostin and renal fibrosis was also reported in previous studies. Histological evaluation was compared between animals with genetic deletion of periostin (Postn null) and wild-type animals after unilateral ureteral obstruction. At day 15, there was an increase in renal fibrosis within kidney tissue samples from wild-type mice. In contrast, less fibrosis area was observed

in Postn null mice. Quantitative analysis of tubulointerstitial fibrosis and tubular dilation provided the same results. Moreover, a significantly higher level of collagen III mRNA expression was found in wild-type mice than in Postn null mice [22]. The same results were also observed in an animal model with polycystic kidney disease. A significant decrease in the percentage of fibrotic areas was found in kidney tissue from animals with loss of periostin expression [129]. In human disease, chronic allograft nephropathy was an appropriate representative of this condition because the main pathway of kidney disease progression after transplantation occurred through the EMT process [130]. Periostin immunohistochemistry showed strong periostin staining in areas with interstitial fibrosis, tubular atrophy and tubular epithelial cells [19]. Periglomeruli and sclerosed glomeruli were also positive for periostin [95]. Moreover, co-staining of periostin and mesenchymal marker vimentin in both glomerular and interstitial areas was detected from serial sections of kidney tissue samples from chronic allograft nephropathy patients [19]. Overall results demonstrated the role of periostin and the renal fibrosis process.

In our study, a significant correlation between total periostin staining score and renal functions was reported. Total periostin staining score was significantly positively correlated with serum creatinine and negatively correlated with eGFR from overall patient and subgroup analysis in LN patients. Supporting our results, periostin immunohistochemistry showed greater intensity and more diffusion of periostin-positive tubulointerstitial areas in LN patients with eGFR below 30 ml/min than in those with eGFR above 60 ml/min [20]. In patients with different nephropathies, quantitative analysis of periostin-positive areas in both glomerular and interstitial areas reported a higher percentage in the group of patients with eGFR below 30 ml/min with statistically significant difference than in the group of patients with eGFR above 60 ml/min. Moreover, a significant negative correlation between periostin mRNA expression and eGFR was also reported in both glomerular and interstitial sections [20]. In our study, subgroup analysis was further evaluated in patients with a low activity index score or a low chronicity index score to find out about the advantage of periostin staining over the

routine staining. Patients were separated into two groups according to their level of total periostin staining score as a low periostin staining score and a high periostin staining score. Interestingly, the results showed that there was a significantly higher level of serum creatinine in patients with a high periostin staining score than in those with a low periostin staining score among patients with a low chronicity index score. In contrast, a significantly lower eGFR was observed in patients with a high periostin staining score than in patients with a low periostin staining score among patients with both a low activity index score and a low chronicity index score. These results demonstrated that higher periostin staining was observed in patients with greater impairment of kidney function. Therefore, periostin staining may be used to predict worsening kidney disease progression rather than routine staining, especially in patients with low active disease or low chronic disease.

The urine periostin level was also evaluated in our study. Urine periostin levels were detected from 23 patients and 11 healthy controls with statistical significance higher in patients than in healthy controls. Subgroup analysis of 17 LN patients and 6 IgA nephropathy patients provided the same results. Even though studies about urine periostin level in patients with lupus nephritis and IgA nephropathy were few and far between. However, there was a significantly higher level of urine periostin reported in both animals and humans with other types of kidney disease. In animals with some part of the kidney removed, there was a significant increase in urine periostin excretion over time after kidney injury. No periostin was detected in urine samples before kidney injury [18]. These results suggest that urine periostin may be used as a biomarker for detecting kidney injury related to chronicity of kidney disease. In human studies, urine periostin was detected in both proteinuric and non-proteinuric chronic kidney disease patients. A significantly higher level of urine periostin was found in both groups of patients than in healthy controls [18]. In chronic allograft nephropathy patients, a significant increase in urine periostin was reported compared with healthy controls and transplant controls. Moreover, a correlation between the percentage of tubulointerstitial

areas and increase in urine periostin was observed, suggesting an association of urine periostin with pathological progression of kidney disease [95]. In our study, there was a tendency of correlation between urine periostin level and interstitial fibrosis as well as tubular atrophy in patients with IgA nephropathy. These results may be due to the higher chronicity index score compared with LN patients. In a recent study of type 2 diabetes patients, there was an increase in urine periostin levels along with a greater degree of albuminuria ranging from normoalbuminuria to microalbuminuria and macroalbuminuria. A significant difference in urine periostin levels was observed between each degree of albuminuria and healthy controls. In addition, there were also significantly higher levels of urine periostin in microalbuminuric and macroalbuminuric patients than in normoalbuminuric patients [96].

At present, the actual mechanism of urine periostin secretion has not been investigated. However, there were some data for supporting these results. According to previous study, immunostaining for periostin in animals with nephrectomy was detected in cytoplasmic tubular epithelial cells, particularly in the apical portion of tubular cells. Moreover, tubular casts and tubular fragments within the tubular lumen were also positive for periostin with more intensity in line with the chronicity of disease [18]. In our study, tubular periostin staining was also a common finding found in most patients. Tubular epithelial cell staining, tubular atrophy staining and tubular cast staining were positive for periostin in both LN patients and IgA nephropathy patients. In patients with diabetic nephropathy, periostin immunostaining was also observed in cytoplasmic tubular epithelial cells in both non-atrophic and atrophic tubular epithelial cells [96]. Tubular periostin staining near the area with interstitial inflammation and fibrosis was detected in kidney tissue from chronic allograft nephropathy patients [95]. In addition, immunofluorescence analysis showed positioning of periostin mainly in distal tubules. No periostin was found in proximal tubules [18]. Overall results demonstrated the possibility of urine periostin secretion from injured renal tubules. Supporting this hypothesis that urine periostin secreted from affected tubules not glomeruli, there was

no statistical difference in urine periostin level between proteinuric and non-proteinuric chronic kidney disease patients [18]. One plausible source of urine periostin could be affected tubular epithelial cells, tubular atrophy and tubular casts. In our study, we further evaluated the correlation between urine periostin level and periostin staining score from kidney tissues. However, no statistically significant correlation between urine periostin levels and periostin staining including tubular epithelial cell staining, tubular atrophy staining and tubular cell cast staining was found. These findings may be resulted from our periostin staining evaluation relied on renal pathologist's consideration to determine positive area and the staining intensity. This method could not provide the exact quantity of periostin expression as we would get from computer analysis. Moreover, limited amount of kidney tissue sample could be obtained from renal biopsy for periostin staining evaluation. Therefore, the correlation between periostin staining and urine periostin level could not be observed. According to the comparison of a quantitative analysis of periostin in kidney tissues and urine samples from the previous study, the immunoblotting analysis showed an increase of periostin in the kidney tissue samples from animals underwent nephrectomy overtime at 2 days, 2 weeks and 4 weeks after the nephrectomy compared with control kidney tissue. In addition, the urine periostin was also increased overtime. These results demonstrated the increment of periostin in kidney tissues together with urine samples overtime after kidney injury [18].

In our study, a subgroup analysis between patients with urine periostin detection and without urine periostin detection was performed. Interestingly, the results showed that there was a significantly greater level of renal function impairment in patients with urine periostin detection than in those without urine periostin detection. Therefore, patients with worsening renal function were characterized by urine periostin. In addition, there was also a significant correlation between urine periostin level and renal functions including serum creatinine, blood urea nitrogen and eGFR in overall patients. Supporting our results, a significant correlation between urine periostin levels and renal functions including serum creatinine, urine protein to creatinine ratio and eGFR was also

observed in patients with chronic allograft nephropathy [95]. In addition, univariate analysis also reported that urine periostin level was correlated with urine albumin to creatinine ratio and eGFR in type 2 diabetes patients. In multiple regression analysis, the increase in urine periostin level was also correlated with increasing urine albumin to creatinine ratio, older age and eGFR reduction [96]. From these results, a higher level of urine periostin may be used as a prognosis of worsening kidney disease. Receiver operating characteristic curves of the urine periostin level were also generated to find out the best cutoff value of the urine periostin level. In previous study, a high sensitivity and specificity of urine periostin for diagnosis of chronic kidney disease patients was reported [18]. In chronic allograft nephropathy patients, the best urine periostin level cutoff value was 0.152 ng/mg, with a sensitivity and specificity of 91.7% and 77.8%, respectively [95]. In diabetes nephropathy patients, the area under the curve for urine periostin level in patients with normoalbuminuria, microalbuminuria and macroalbuminuria was also statistically significantly different from the reference line. A moderate to high sensitivity and specificity for diagnosing diabetic nephropathy was also reported [96]. These data demonstrated the value of urine periostin measurement for diagnosing various types of chronic kidney disease. In our study, subgroup analysis in LN and IgA nephropathy patients reported the best urine periostin level cutoff value to be 2.098 ng/mg (sensitivity 45.9% and specificity 88.0%) and 5.775 ng/mg (sensitivity 46.2% and specificity 94.0%), respectively. The accuracy was 70.1% and 84.1%, respectively. In our study, urine periostin was also detected in patients with greater impairment of renal functions. The median serum creatinine and eGFR were 0.9 mg/dl and 74.79 ml/min/1.73m^2, respectively. Urine periostin was not detected in 54% of patients with median serum creatinine at 0.7 mg/dl and eGFR at 97.18 ml/min/1.73m^2. According to our results, urine periostin may be used as a prognosis of disease progression in LN and IgA nephropathy patients.

In our study, serum periostin level was also measured in both patients and healthy controls. No previous study has investigated the serum periostin level in patients

with lupus nephritis and IgA nephropathy. The results showed that there was no statistically significant difference between the serum periostin level in patients and healthy controls. Subgroup analysis in LN patients and IgA nephropathy patients was the same. In contrast, there was a statistically significant difference in urine periostin level between kidney disease patients and healthy controls. These results suggested that periostin may be used as a urinary biomarker for detecting kidney injury in patients with LN and IgA nephropathy rather than serum periostin measurement. In addition, no correlation was found between serum periostin level and urine periostin level, suggesting the possible source of urine periostin from kidney tissue. We further analyzed the correlation between serum periostin and other variables. According to histopathological features, there was a significant correlation between serum periostin level and glomerular cell proliferation in IgA nephropathy patients. In previous studies, an association between periostin and cell proliferation was reported. After incubating mesangial cells with different concentrations of periostin, there was a significant increase in cell proliferation compared with controls (absence of periostin), with the highest cell proliferation being 10 ng/ml periostin [20]. The same results were found in autosomal dominant polycystic kidney disease cells. There was a significant increase in the number of cells compared with controls [21]. In a polycystic kidney disease animal model, immunohistochemistry of a cell proliferation marker showed fewer cells with a proliferation in kidney tissue samples from animals without periostin expression. In addition, quantitative analysis of proliferating cell numbers reported the same. There was a significantly lower number of cells with proliferation in animals without periostin expression, suggesting an effect of periostin on cell proliferation [129]. In our study, we also investigated the correlation between serum periostin level and other variables. In LN patients, serum periostin level was significantly correlated with body weight. In IgA nephropathy patients, there was a significant correlation between serum periostin level and diastolic blood pressure as well as serum albumin. These results suggested that there were other factors that were not related to the pathology of kidney disease that affected serum periostin level.

In our study, we also assessed that whether periostin measurement and other variables were related to response to therapy after 6 months of treatment. There was no statistical difference in baseline renal pathology including activity index and chronicity index between patients with response and non-response to therapy. Other variables, including characteristic data, renal parameters, treatment, urine periostin level and serum periostin level at baseline were not different between patients with response and non-response to therapy. In our study we also compared urine periostin level before and after 6 months of treatment. The results found that urine periostin level was significantly lower after 6 months of treatment in patients with response to therapy. No significant difference was observed in patients with non-response to therapy. These results indicated the potential of urine periostin measurement as a biomarker for monitoring response to therapy. To the best of our knowledge, this is the first study that has evaluated urine periostin level and response to treatment in human with kidney disease. Even though studies about periostin and response to therapy were few and far between, there was one study that investigated the periostin mRNA expression and the progression of kidney disease after treatment. In animals with hypertensive nephropathy, there was a significantly lower periostin mRNA expression within kidney tissue samples from animals with regressive hypertensive nephropathy than from those with progressive hypertensive nephropathy after treatment for 4 weeks [19]. In addition, the role of periostin as a therapeutic target was also observed in animals with hypertensive nephropathy. Histological evaluation from kidney tissue showed less fibrosis and tubular dilation in animals with periostin mRNA interference. Quantifications of glomerulosclerosis, perivascular fibrosis, vascular hypertrophy and tubular dilation had also decreased. In addition, a significant decline of proteinuria was reported in animals with periostin mRNA interference compared with those with non-interference [22]. According to our study, there was a significant reduction of urine periostin level in patients with response to therapy. However, there was a limitation due to the small sample size that could be obtained for evaluation. A larger sample size should be

further investigated to find out the possibility of using urine periostin measurement for the prognosis and monitoring of response to therapy.

CHAPTER VI

CONCLUSION

This study investigated the periostin levels in kidney tissue, urine and serum samples from patients with lupus nephritis and IgA nephropathy. It was conducted from April 2013 to February 2015 at the Department of Medicine, Phramongkutklao Hospital, Bangkok, Thailand. A total of 50 patients were included in this study. There were 37 and 13 patients diagnosed with lupus nephritis and IgA nephropathy, respectively. Kidney tissue, urine and serum samples were collected for measuring periostin. In kidney tissue samples, activity index, chronicity index and periostin staining score were assessed by a renal pathologist. For the activity index score, glomerular cell proliferation and hyaline thrombi or wire loop were the most common findings in overall patients. The same tendency was also reported in subgroup analysis of LN and IgA nephropathy patients with median total activity index scores of 4 (2, 6) and 3 (2, 5), respectively. For the chronicity index score, interstitial fibrosis and tubular atrophy were the most common findings in overall patients. Half of the overall patients also presented glomerular sclerosis. The same was reported in both LN and IgA nephropathy patients with median total chronicity index scores of 2 (0, 4) and 4 (3, 7), respectively. According to periostin immunohistochemistry in kidney tissue samples, the results showed that periostin was not detected in control kidney tissue. In contrast, periostin staining was found in glomerular, interstitial, tubular and vascular areas from patients' kidney tissues. Periostin staining analysis reported that the most common area with positive periostin was tubular including tubular epithelial cells, tubular cell casts and tubular atrophy. There was also positive periostin in areas with interstitial fibrosis, sclerosed glomeruli and periglomeruli. The same results were also observed in subgroup analysis of LN patients. For IgA nephropathy patients, positive periostin staining in periglomeruli, global sclerosis,

interstitial fibrosis, vascular fibrosis, tubular epithelial cells and tubular cell casts was also observed in more than half of the patients.

The correlation between periostin staining and renal pathology from overall patients showed that total periostin staining score was significantly correlated with chronicity index score ($r = 0.527$, p-value < 0.001). A positive correlation was also reported with renal pathology score within the chronicity index including segmental sclerosis, fibrous crescent, interstitial fibrosis and tubular atrophy. In addition, total periostin staining score was significantly correlated with renal functions including serum creatinine ($r = 0.361$, p-value $= 0.010$) and eGFR ($r = -0.373$, p-value $= 0.008$). The same results were also reported in subgroup analysis of LN patients. In our study, we also investigated the relevance of high/low periostin staining score levels in patients with a low activity index score or a low chronicity index score. Worsening of renal functions was observed in patients with a high periostin staining score. In patients with a low activity index score, there was a significantly lower level of eGFR in patients with a high periostin staining score. In patients with a low chronicity index score, there was a significantly higher level of serum creatinine and lower level of eGFR reported in patients with a high periostin staining score. These results demonstrated the favorable role of periostin staining in the prognosis of kidney disease progression rather than routine staining. In our study, kidney tissue samples were also evaluated for periostin and TGF-β mRNA expression. No significant difference in periostin mRNA expression was found in kidney tissue from patients compared with control tissues. In contrast, there was a significantly higher level of TGF-β mRNA expression in kidney tissues from patients than in control tissues. These results indicated the relevance of a fibrosis mediator and kidney injury.

According to urine periostin analysis, urine periostin was detected in 23 out of 50 patients and 11 out of 50 healthy controls. A significantly higher urine periostin level was found in patients than in healthy controls. Subgroup analysis of lupus nephritis patients

and IgA nephropathy patients reported the same. Further evaluation between patients with and without urine periostin detection reported worsening renal function in patients with urine periostin detection. In addition, there was a significant correlation between urine periostin level and renal functions including serum creatinine, blood urea nitrogen and eGFR. These results suggested the possibility of using urine periostin measurement for the prognosis of disease progression.

In our study, the serum periostin level was also evaluated. There was no statistically significant difference in serum periostin level between patients and healthy controls. In addition, no correlation was found between serum periostin level and urine periostin level. However, a correlation between serum periostin level and some renal pathological features was observed such as segmental sclerosis in lupus nephritis patients and glomerular cell proliferation in IgA nephropathy patients. There were also other variables that affected periostin level including body weight, diastolic blood pressure and serum albumin. After 6 months of treatment, there was no statistical difference in baseline renal pathology, characteristic data, renal parameters, treatment, urine periostin level and serum periostin level between patients with response and those with non-response to therapy. However, there was a significant decrease in urine periostin level in patients with response to therapy after 6 months of treatment.

In conclusion from overall results in this study, periostin may be a promising tissue biomarker that is related to chronic kidney disease progression and kidney functions. Periostin staining may be used to predict worsening kidney disease progression rather than routine staining, especially in patients with low active disease or low chronic disease. Urine periostin can distinguish patients with LN and IgA nephropathy from normal controls rather than serum periostin. Urine periostin measurement may be used for the prognosis of disease progression in LN and IgA nephropathy patients. A possibility of using urine periostin measurement for monitoring response to therapy after 6 months of treatment was also observed.

Limitations of the present study

1. In this study, normal kidney tissue sample from healthy controls could not be obtained in clinical practice. Therefore, kidney tissue samples from renal cell carcinoma patients were collected instead. However, only normal kidney tissue sections from renal cell carcinoma patients which were confirmed by renal pathologist were used.
2. Kidney tissue samples for mRNA expression analysis could be obtained from only 13 out of 50 patients. The results cannot be extrapolated to overall patients from small sample sizes.
3. There was a small sample size for evaluating urine periostin measurement and response to therapy after 6 months of treatment.
4. Periostin staining was measured from the intensity of the area with periostin positive by a renal pathologist. Unlike computer analysis, periostin staining could not provide exact quantity of periostin expression.

Recommendations

1. More kidney tissue samples from patients and controls should be obtained for periostin mRNA expression analysis.
2. The measurement of EMT markers should be investigated together with periostin at both protein and gene level in kidney tissue samples to find out more about the relevance of periostin and the fibrosis process.
3. More urine samples should be collected to evaluate the potential of urine periostin measurement for monitoring response to therapy.
4. The computer program analysis should be used for periostin staining evaluation which provides exact quantity of periostin expression in kidney tissue samples.

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APPENDICES

Appendix A

Ethical Approval Documents

RL 01_2555

ที่ IRB/RTA 489/2556

คณะอนุกรรมการพิจารณาโครงการวิจัย กรมแพทย์ทหารบก

317 ถนนราชวิถี เขต ราชเทวี กรุงเทพฯ 10400

รหัสโครงการ: Q023hh/55

ชื่อโครงการวิจัย : "การศึกษาความสัมพันธ์ของตัวชี้วัดเพอริออสตินกับพยาธิสภาพของไตในผู้ป่วยโรคไตวายเรื้อรัง"
[The correlation of periostin and renal pathology in chronic kidney disease patients.]

เลขที่โครงการวิจัย : -

ชื่อผู้วิจัยหลัก: ภญ.ปิย์ภัทรา วันทนาศิริ นักศึกษาปริญญาเอก คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สังกัดหน่วยงาน : คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สถานที่ทำการวิจัย: หน่วยโรคไต กองอายุรกรรม โรงพยาบาลพระมงกุฎเกล้า

เอกสารรับรอง :

(1) โครงร่างการวิจัยฉบับภาษาไทย ฉบับที่ 3 วันที่ 21 มี.ค. 56
(2) แบบบันทึก ก. ข้อมูลเบื้องต้น และ ข. แบบการประเมินผลเพอริออสติน ฉบับที่ 3 วันที่ 21 มี.ค. 56
(3) เอกสารชี้แจงข้อมูลแก่ผู้เข้าร่วมโครงการวิจัย ฉบับที่ 3 วันที่ 21 มี.ค. 56
(4) หนังสือแสดงเจตนายินยอมเข้าร่วมการวิจัย ฉบับที่ 3 วันที่ 21 มี.ค. 56
(5) ประวัติย่อ ภญ.ปิย์ภัทรา วันทนาศิ ฉบับที่ 1 วันที่ 28 พ.ย. 55
(6) ประวัติย่อ รศ.ดร.พรอนงค์ อร่ามวิทย์ ฉบับที่ 1 วันที่ 28 พ.ย. 55
(7) ประวัติย่อ ร.อ.บัญชา สถิระพจน์ ฉบับที่ 1 วันที่ 28 พ.ย.55
(8) ประวัติย่อ พ.ต.สุเมธ เติมมธุระพจน์ ฉบับที่ 1 วันที่ 28 พ.ย. 55
(9) ประวัติย่อ ร.ท.นพ.มงคล เจริญพิทักษ์ชัย ฉบับที่ 1 วันที่ 28 พ.ย. 55

ขอรับรองว่าโครงการดังกล่าวข้างต้นได้ผ่านการพิจารณารับรองจากคณะอนุกรรมการพิจารณาโครงการวิจัย กรมแพทย์ทหารบก ว่าสอดคล้องกับปฏิญญาเฮลซิงกิ และแนวปฏิบัติ ICH GCP

วันที่รับรองด้านจริยธรรมของโครงร่างการวิจัย: 28 มีนาคม 2556
วันสิ้นสุดการรับรอง: 27 มีนาคม 2557
ความถี่ของการส่งรายงานความก้าวหน้าของการวิจัย: รายงานความก้าวหน้าทุก 1 ปี

.....
พันเอกหญิง เยาวนา ธนะพัฒน์
ประธานคณะอนุกรรมการพิจารณาโครงการวิจัย พบ.

.....
พันเอกสหพล อนันต์นำเจริญ
เลขานุการและอนุกรรมการพิจารณาโครงการวิจัย พบ.

ที่ IRBRTA.1163/2556

คณะอนุกรรมการพิจารณาโครงการวิจัย กรมแพทย์ทหารบก

317 ถนนราชวิถี เขต ราชเทวี กรุงเทพฯ 10400

รหัสโครงการ: Q023h/55

ชื่อโครงการวิจัย : การศึกษาความสัมพันธ์ของตัวชี้วัดเพอริออสตินกับพยาธิสภาพของไตในผู้ป่วยโรคไตวาย

ชื่อผู้วิจัยหลัก: ภญ. ปิย์ภัทรา วันทนาศิริ

สังกัดหน่วยงาน : คณะเภสัชศาสตร์ จุฬาลงกรณ์มหาวิทยาลัย

สถานที่ทำการวิจัย: หน่วยโรคไต กองอายุรกรรม โรงพยาบาลพระมงกุฎเกล้า

เอกสารที่ทบทวน : **ส่วนแก้ไขเพิ่มเติมโครงร่างการวิจัย**

1. โครงร่างการวิจัย ฉบับที่ 4 วันที่ 29 กรกฎาคม 2556
   - หัวข้อที่ 12 วิธีการดำเนินการวิจัย
   - หัวข้อที่ 14 การวิเคราะห์ข้อมูล
2. เอกสารชี้แจงข้อมูลแก่ผู้เข้าร่วมโครงการวิจัย ฉบับที่ 4 วันที่ 29 กรกฎาคม 2556
   - เพิ่มเติมในหัวข้อ ระยะเวลาที่ท่านจะต้องเข้าร่วมโครงการวิจัยและจำนวนครั้งที่นัด

คณะอนุกรรมการพิจารณาโครงการวิจัย กรมแพทย์ทหารบก ได้พิจารณาส่วนแก้ไขเพิ่มเติมโครงร่างการวิจัยแล้ว จึงขอตอบรับและรับรองเอกสารดังกล่าว ผู้วิจัยสามารถดำเนินการตามโครงร่างการวิจัยที่แก้ไขเพิ่มเติมได้ตั้งแต่วันที่คณะอนุกรรมการฯ รับรอง

วันที่รับรองส่วนแก้ไขเพิ่มเติมโครงร่างการวิจัย 14 สิงหาคม 2556

พันเอกหญิง.....
(เยาวนา ธนะพัฒน์)
ประธานคณะอนุกรรมการพิจารณาโครงการวิจัย พบ.

พันเอก.....
(สหพล อนันต์นำเจริญ)
เลขานุการและอนุกรรมการพิจารณาโครงการวิจัย พบ.

Appendix B

Kidney Tissue Staining Protocols

Hematoxylin and Eosin (H&E) Staining

Protocol for H&E staining

1. Deparaffinize with xylene for 5 minutes (2 times).
2. Hydrate with isopropyl alcohol for 1 minute (2 times).
3. Hydrate with 95% alcohol for 1 minute (2 times).
4. Wash with running tap water for 2 minutes.
5. Stain nucleus with Mayer's hematoxylin stain for 10 minutes.
6. Wash with running tap water for 3 minutes.
7. Stabilize with saturated lithium carbonate for 2 seconds.
8. Wash with running tap water for 2 minutes.
9. Wash with distilled water for 10 seconds.
10. Pre-eosin stain with 95% ethyl alcohol for 2 seconds.
11. Stain cytoplasm and nucleolus with Eosin stain for 10 seconds.
12. Dehydrate with 95% ethyl alcohol for 1 minute (2 times).
13. Dehydrate with isopropyl alcohol for 1 minute (2 times).
14. Clear with xylene for 2 minutes (2 times).

H&E stain evaluation

- Nuclei — Blue
- Cytoplasm — Pink to red
- Most other tissue structure — Pink to red

Masson's Trichrome Staining

Protocol for Masson's Trichrome staining

1. Deparaffinize with xylene for 5 minutes (2 times).
2. Hydrate with absolute alcohol, 95% alcohol and distilled water.
3. Immerse slide in Bouin's solution at 56ºC for 1 hour.
4. Wash with running tap water until all yellow disappears.
5. Immerse slide in distilled water.
6. Immerse slide in Weigert's iron hematoxylin for 4-5 minutes and wash with running tap water for 10 minutes.
7. Wash with distilled water.
8. Immerse slide in Biebrich scarlet-Acid fuchsin solution for 20 minutes.
9. Wash with distilled water.
10. Immerse slide in Phosphomolybdic-Phosphotunstic acid solution for 5 minutes.
11. Wash with distilled water.
12. Immerse slide in Aniline blue solution for 5 minutes and wash with distilled water.
13. Immerse slide in 1% Acetic acid solution for 6-10 seconds and washing with distilled water.
14. Dehydrate with 95% alcohol and absolute alcohol.
15. Clear with xylene and mount with permount.

Masson's Trichrome stain evaluation

- Nuclei	Black
- Cytoplasm, keratin, muscle fiber Intercellular fiber	Red
- Collagen	Blue

Periodic Acid-Schiff Staining

Protocol for Periodic Acid-Schiff staining

1. Deparaffinize with xylene for 5 minutes (2 times).
2. Hydrate with absolute alcohol, 95% alcohol and distilled water.
3. Immerse slide in 1% Periodic solution for 10 minutes and wash with distilled water.
4. Stain with Schiff's Leuco-fuchsin solution for 15 minutes.
5. Wash with running tap water for 10 minutes or until tissue become pink.
6. Counterstain with Mayer's hematoxylin solution for 3 minutes and wash with running tap water.
7. Immerse slide in Lithium carbonate (bluing solution).
8. Wash with running tap water.
9. Dehydrate with 95% alcohol and absolute alcohol.
10. Clear with xylene and mount with permount.

Periodic Acid-Schiff stain evaluation

- Glycogen, Fungus — Red / Purple
- Nucleus — Blue

Immunohistochemistry for Periostin Staining

Protocol for immunohistochemistry for periostin staining

1. Deparaffinize with EZ prep (Ventana, USA, Ref#950-102).
2. Rinse slide with Reaction Buffer (Ventana, Cat#950-300).
3. Add UV INHIBITOR (Ventana, Ref#760-500) incubate for 4 minutes.
4. Rinse slide with Reaction Buffer.
5. Add Protease 2 (Ventana, Ref#760-2019) incubate for 10 minutes.
6. Rinse slide with Reaction Buffer.
7. Add PRIMARY ANTIBODY dilution 1:1000 (Abcam, Cambridge, UK, Cat#ab14041) incubate for 32 minutes.
8. Rinse slide with Reaction Buffer.
9. Add UV HRP UNIV MULT (Ventana, Ref#760-500) incubate for 8 minutes.
10. Rinse slide with Reaction Buffer.
11. Add UV DAB และ UV DAB H_2O_2 (Ventana, Ref#760-500) incubate for 8 minutes.
12. Rinse slide with Reaction Buffer.
13. Add UV COPPER (Ventana, Ref#760-500) incubate for 4 minutes.
14. Rinse slide with Reaction Buffer.
15. Add HEMATOXYLIN II (Ventana, Cat#790-2208) (Counterstain) incubate for 12 minutes.
16. Rinse slide with Reaction Buffer.
17. Add BLUING REAGENT (Ventana, Cat#760-2037) (Post Counterstain) incubate for 12 minutes.
18. Rinse slide with Reaction Buffer.

Immunohistochemistry for periostin

- Periostin location — Brown

Appendix C

Periostin mRNA Expression by Real-time Polymerase Chain Reaction

RNA extraction with RNeasy Mini Kit (Qiagen)

1. Disruption kidney tissue with β-mercaptoethanol in Buffer RLT by using micropestle.
2. Centrifuge lysate at 13,000 rpm for 3 minutes and pipet supernatant (lysate) into a new microcentrifuge tube.
3. Add 70% ethanol into cleared lysate, mix immediately and transfer the sample and any precipitation into RNeasy spin column.
4. Centrifuge at 13,000 rpm for 3 minutes (2 times).
5. Add 350 µl Buffer RW1, centrifuge at 12,000 rpm for 1 minute and discard the flow-through.
6. Add DNase I incubation mix 80 µl directly to the RNeasy spin column membrane and place on the benchtop (20-30ºC) for 20 minutes.
7. Add 350 µl Buffer RW1 to the RNeasy spin column, centrifuge at 12,000 rpm for 1 minute and discard the flow-through.
8. Add 500 µl Buffer RPE to the RNeasy spin column, centrifuge at 12,000 rpm for 1 minute and discard the flow-through.
9. Add 500 µl Buffer RPE to the RNeasy spin column, centrifuge at 12,000 rpm for 2 minutes and discard the collection with the flow-through and place RNeasy spin column in new 2 ml collection tube.
10. Centrifuge at 12,000 rpm for 1 minute.
11. Discard the collection with the flow-through and place RNeasy spin column in new 1.5 ml collection tube.
12. Add 30 µl RNase free water (for control kidney tissues) and 25 µl RNase free water (for kidney tissues from patients).

13. Incubate 1 minute at room temperature and centrifuge at 12,000 rpm for 1 minute to elute RNA.
14. Measure RNA concentration.

RNA convertion to cDNA with MonsterScript 1st – Strand cDNA Synthesis (Qiagen)

1. Dilute RNA with RNase free water.
2. Add Random primers 2 µl.
3. Incubate at 65°C for 1 minute.
4. Chill on ice for 1 minute.
5. Add Monster MonsterScript 5X cDNA Premix 4 µl and MonsterScript Reverse Transcriptase 1 µl.
6. Incubate at 37°C for 5 minutes.
7. Incubate at 42°C for 5 minutes and then 60°C for 40 minutes.
8. Terminate the reaction by heating at 90°C for 5 minutes.
9. Chill on ice 1 minute and spin down.
10. The cDNA can be used immediately or store at -20°C before future using.

Prepare PCR reaction mix with All-in-One qPCR Mix (GeneCopoeia)

Prepare PCR reaction mix as describe in the Table below in PCR reaction tubes.

Reagent	Volume
2X All-in-One qPCR Mix	10 µl
Gene primer -β-actin (NM_001101.3) -Periostin (NM_006475.1) -TGF-β (NM_000660.3)	1 µl
cDNA template	2 µl
50X ROX Reference Dye	0.4 µl
ddH2O	6.6 µl
Total	20 µl

Real-time Polymerase Chain Reaction

Real-time PCR was performed by using the 7500 Real Time PCR System (Applied Biosystems, Foster City, CA) as a condition describe in the Table below.

Cycles	Steps	Temperature	Time	Detection
1	Initial denaturation	95°C	10 min	No
40	Denaturation	95°C	10 sec	No
	Annealing	60°C	20 sec	No
	Extension	72°C	35 sec	Yes

Appendix D

Enzyme-linked Immunosorbent Assay for Urine Periostin

Plate preparation

1. Coat 96-well microplate overnight with 1 µg/ml (0.1 µg per well) of anti-periostin antibody (R&D Systems, Minneapolis, MN, Cat#AF3548), diluted in 0.006 M Carbonate buffer, pH 9.6. Incubate the plate for 24 hours at 4°C.
2. Wash the plate three times with 0.05% Tween 20 in phosphate-buffered saline (PBS).
3. Block the plate with Reagent Diluent (0.5% BSA + 0.5% Casein in PBS, pH 7.4) 300 µl for 2 hours at room temperature.
4. Repeat the wash as in step 2. The plate is now ready for sample addition.

Assay Procedure

1. Add 100 µl of all standard serial dilutions (R&D Systems, Recombinant Human Periostin/OSF-2, Cat#3548-F2-050) and urine samples to the 96-well plate and incubate for 2 hours at 4°C.
2. Wash the plate three times with 0.05% Tween 20 in PBS.
3. Add 100 µl of rabbit polyclonal antibodies to periostin (Abcam, Cambridge, UK, 1:1000, Cat# ab14041) and incubate for 1 hour at room temperature.
4. Wash the plate three times with 0.05% Tween 20 in PBS.
5. Add 100 µl of horseradish peroxidase conjugated antibody (GE Healthcare Bio-Sciences, USA, 1:200, Cat#NA934-100µl) to each well. Cover the plate and incubate for 20 minutes at room temperature.
6. Wash the plate three times with 0.05% Tween 20 in PBS.

7. Add 100 µl of substrate solution (R&D Systems, Cat#DY999) to each well. Incubate for 20 minutes at room temperature.
8. Add 50 µl of stop solution (R&D Systems, Cat#DY994) to each well. Gently tap the plate to ensure thorough mixing.
9. Periostin absorbances were calculated by taking measurements at 450 nm. Periostin concentrations were calculated based on a log-transformed standard curve.

Appendix E

Enzyme-linked Immunosorbent Assay for Serum Periostin

Plate preparation

1. Coat 96-well microplate overnight with 1 µg/ml (0.1 µg per well) of anti-periostin antibody (R&D Systems, Minneapolis, MN, Cat#AF3548), diluted in 0.006 M Carbonate buffer, pH 9.6. Incubate the plate for 24 hours at 4°C.
2. Wash the plate three times with 0.05% Tween 20 in PBS.
3. Block the plate with Reagent Diluent (0.5% BSA + 0.5% Casein in PBS, pH 7.4) 300 µl for 2 hours at room temperature.
4. Repeat the wash as in step 2. The plate is now ready for sample addition.

Assay Procedure

1. Add 100 µl of all standard serial dilutions (R&D Systems, Recombinant Human Periostin/OSF-2, Cat#3548-F2-050) and serum samples (dilute 1:50) to the 96-well plate and incubate for 2 hours at 4°C.
2. Wash the plate three times with 0.05% Tween 20 in PBS.
3. Add 100 µl of rabbit polyclonal antibodies to periostin (Abcam, Cambridge, UK, 1:1000, Cat# ab14041) and incubate for 1 hour at room temperature.
4. Wash the plate three times with 0.05% Tween 20 in PBS.
5. Add 100 µl of horseradish peroxidase conjugated antibody (GE Healthcare Bio-Sciences, USA, 1:200, Cat#NA934-100µl) to each well. Cover the plate and incubate for 20 minutes at room temperature.
6. Wash the plate three times with 0.05% Tween 20 in PBS.
7. Add 100 µl of substrate solution (R&D Systems, Cat#DY999) to each well. Incubate for 20 minutes at room temperature.

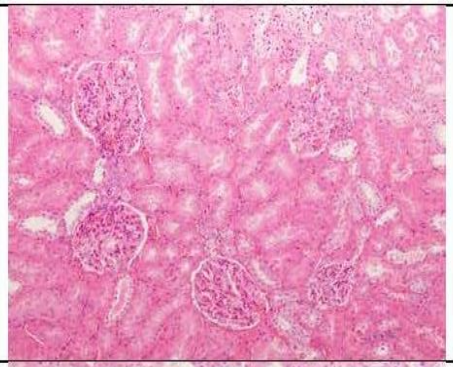
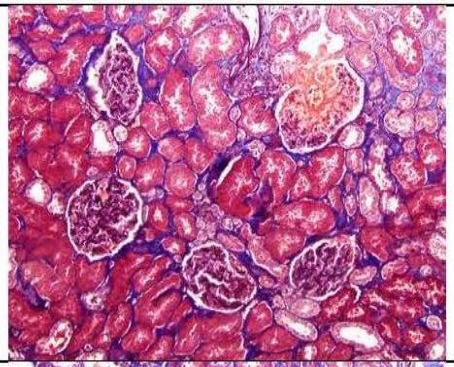
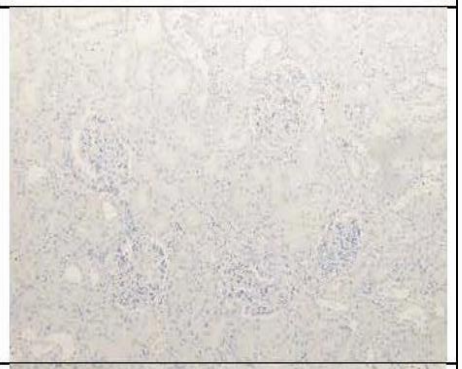
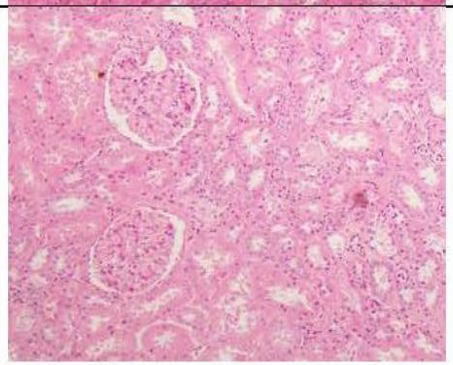
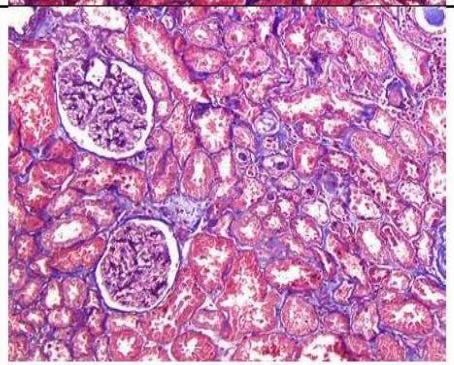
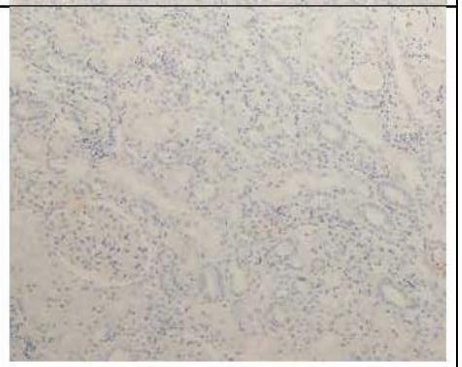
8. Add 50 µl of stop solution (R&D Systems, Cat#DY994) to each well. Gently tap the plate to ensure thorough mixing.
9. Periostin absorbances were calculated by taking measurements at 450 nm. Periostin concentrations were calculated based on a log-transformed standard curve.

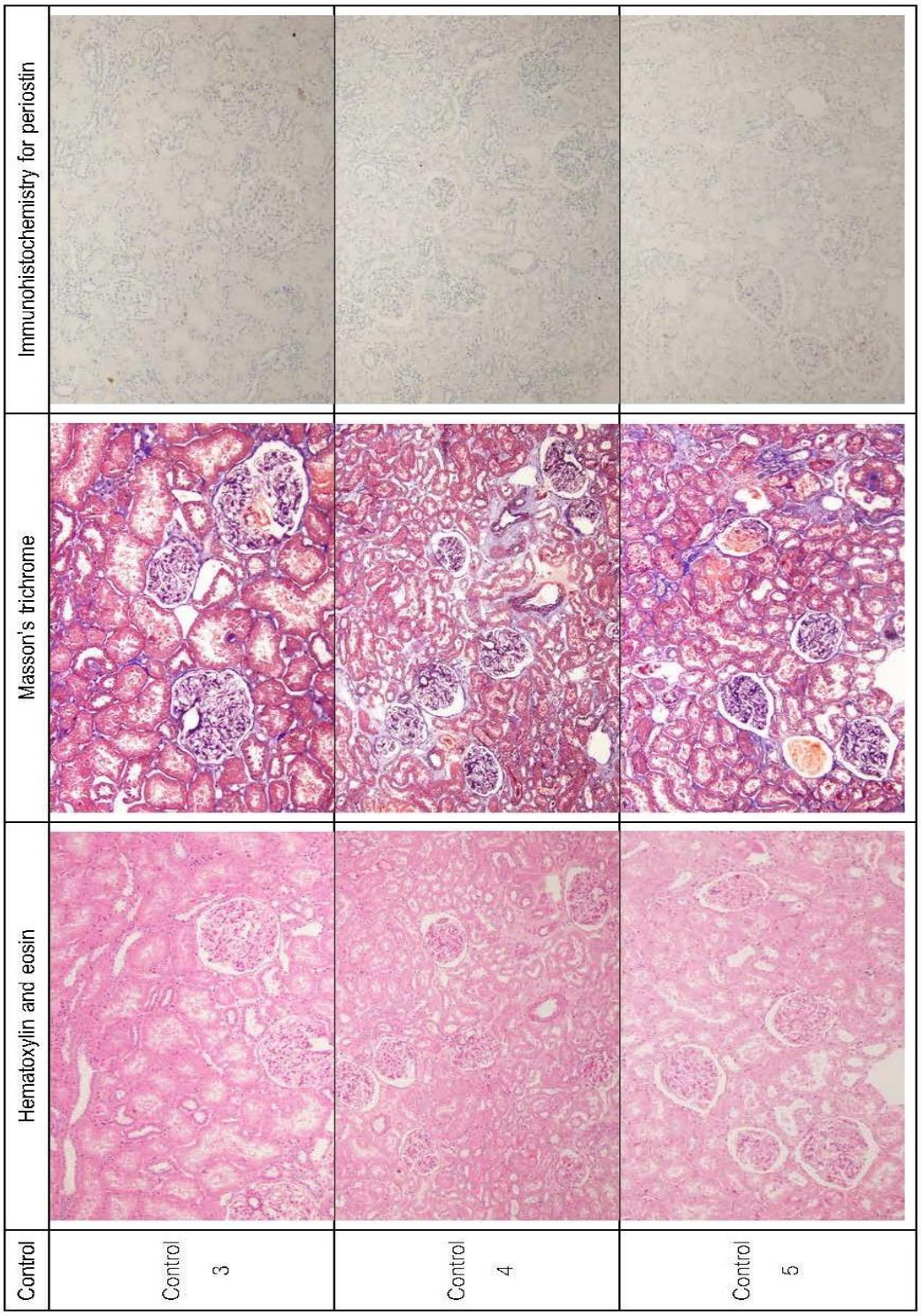
Appendix F

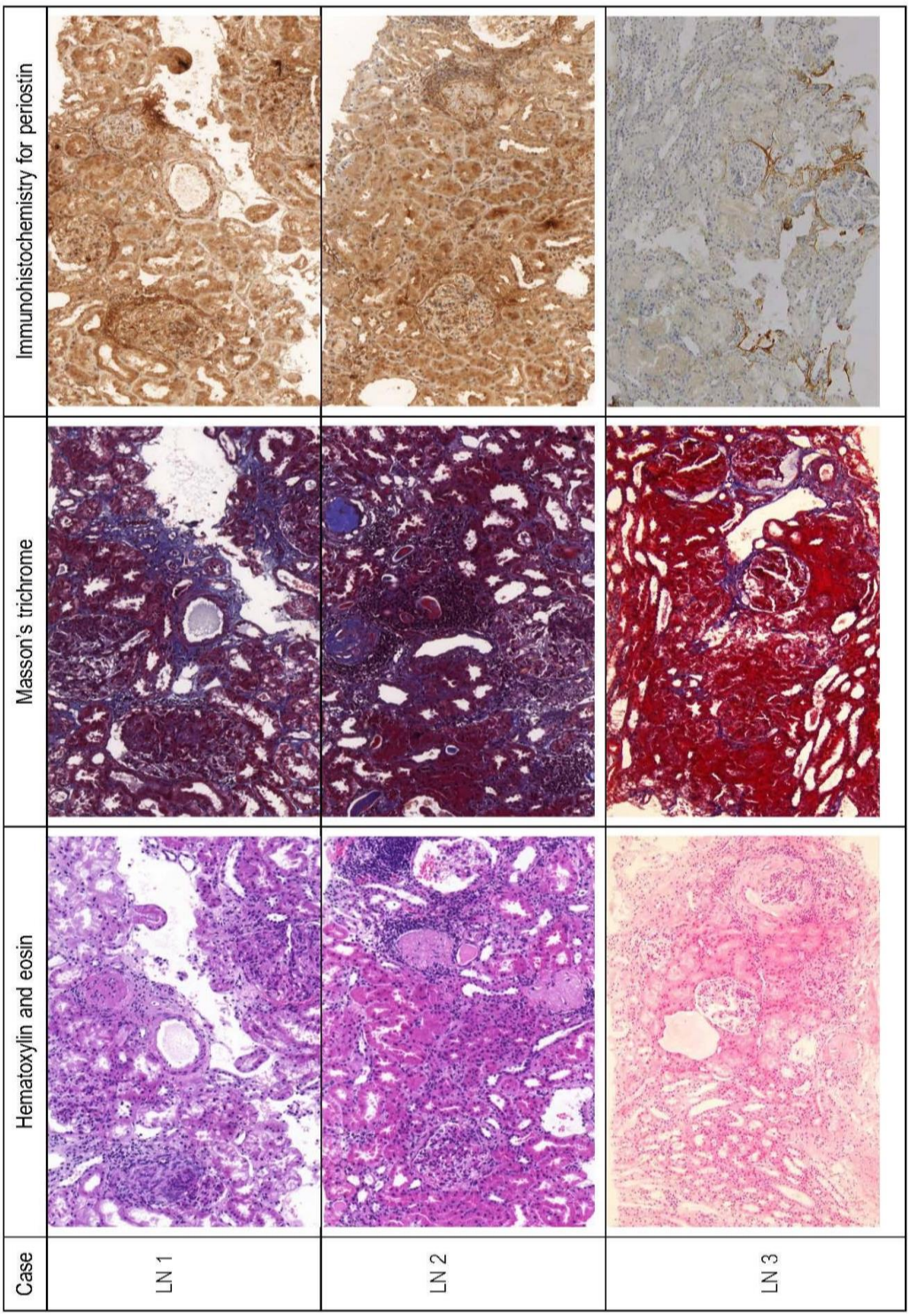
Hematoxylin and Eosin, Masson's Trichrome and Immunohistochemistry for Periostin Stain in Kidney Tissue Samples

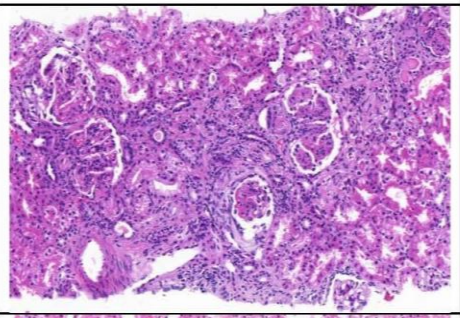
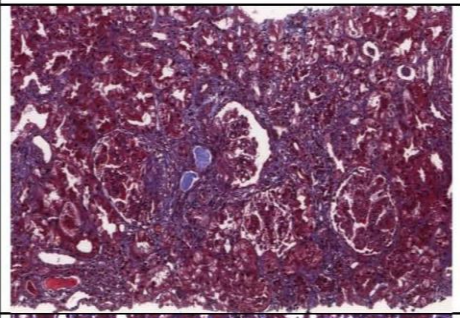
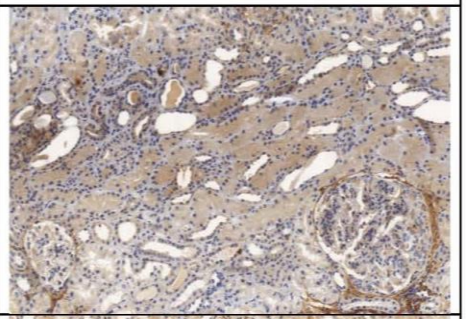
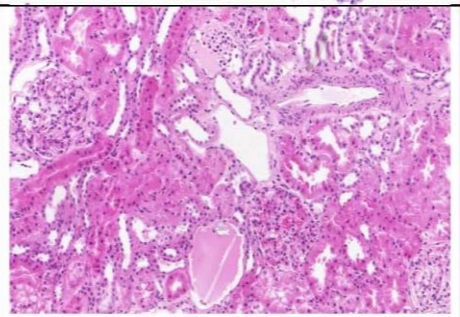
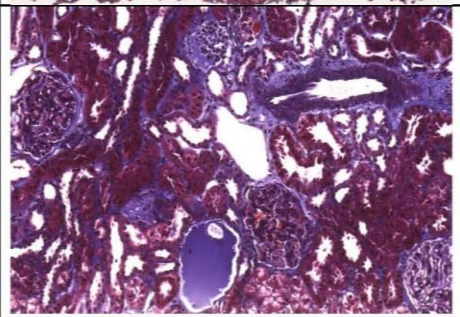
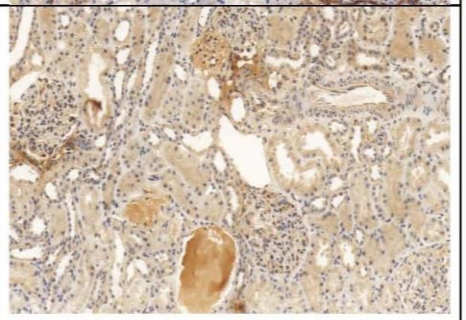
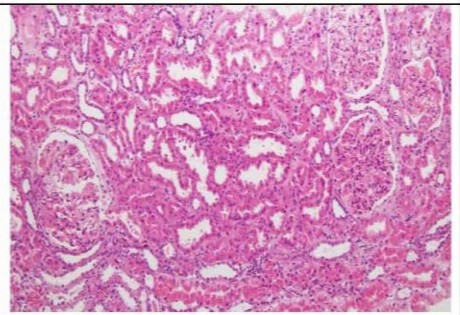
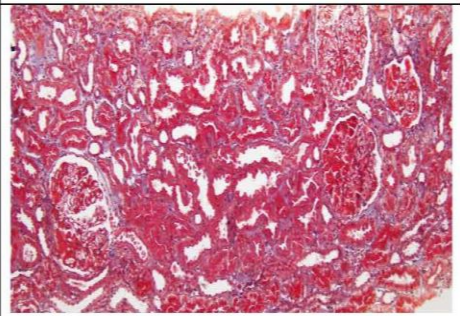
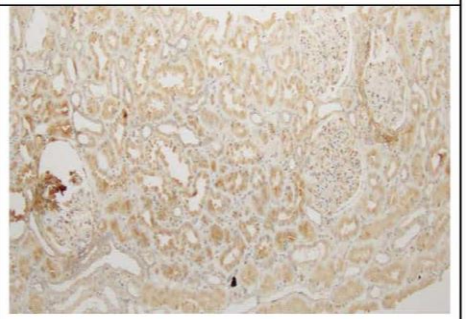
Appendix F

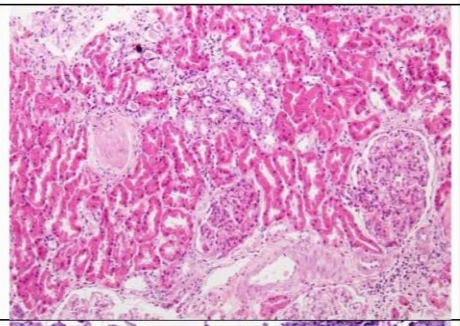
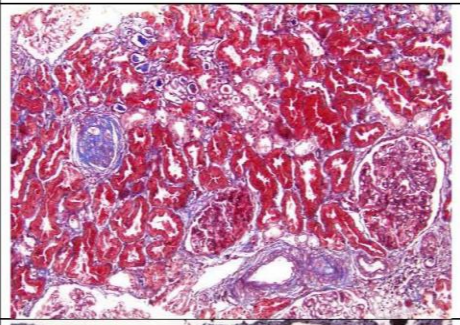
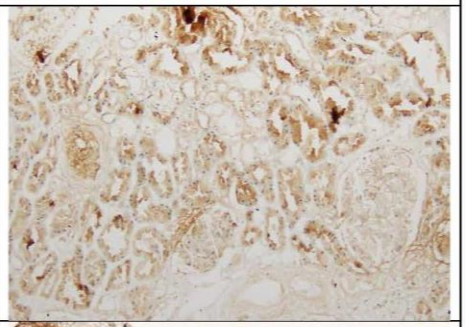
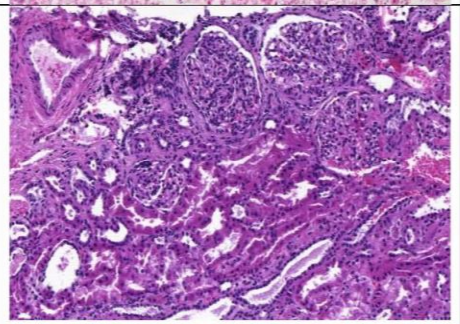
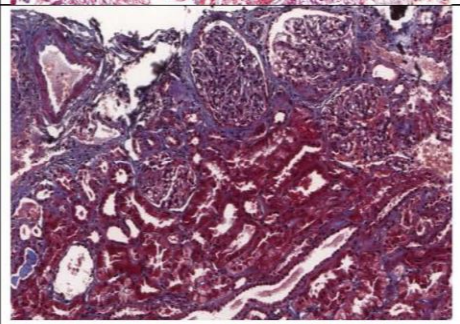
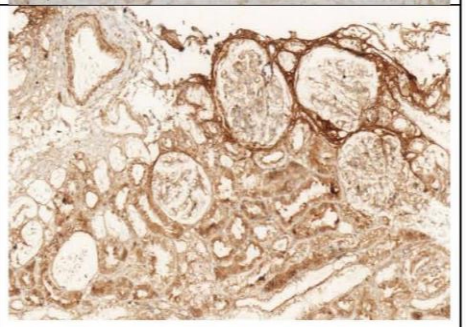
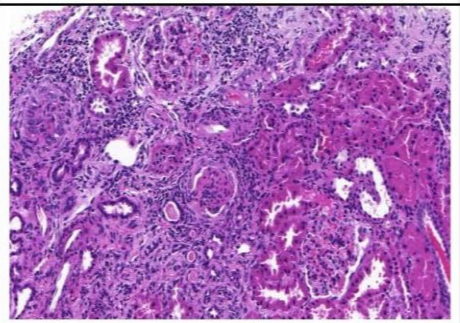
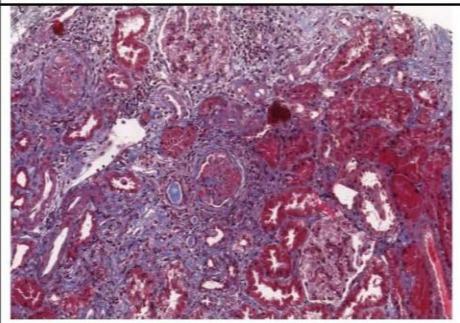
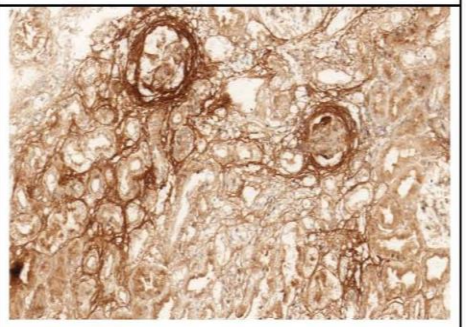
Hematoxylin and eosin, Masson's trichrome and Immunohistochemistry for periostin stain in kidney tissue samples

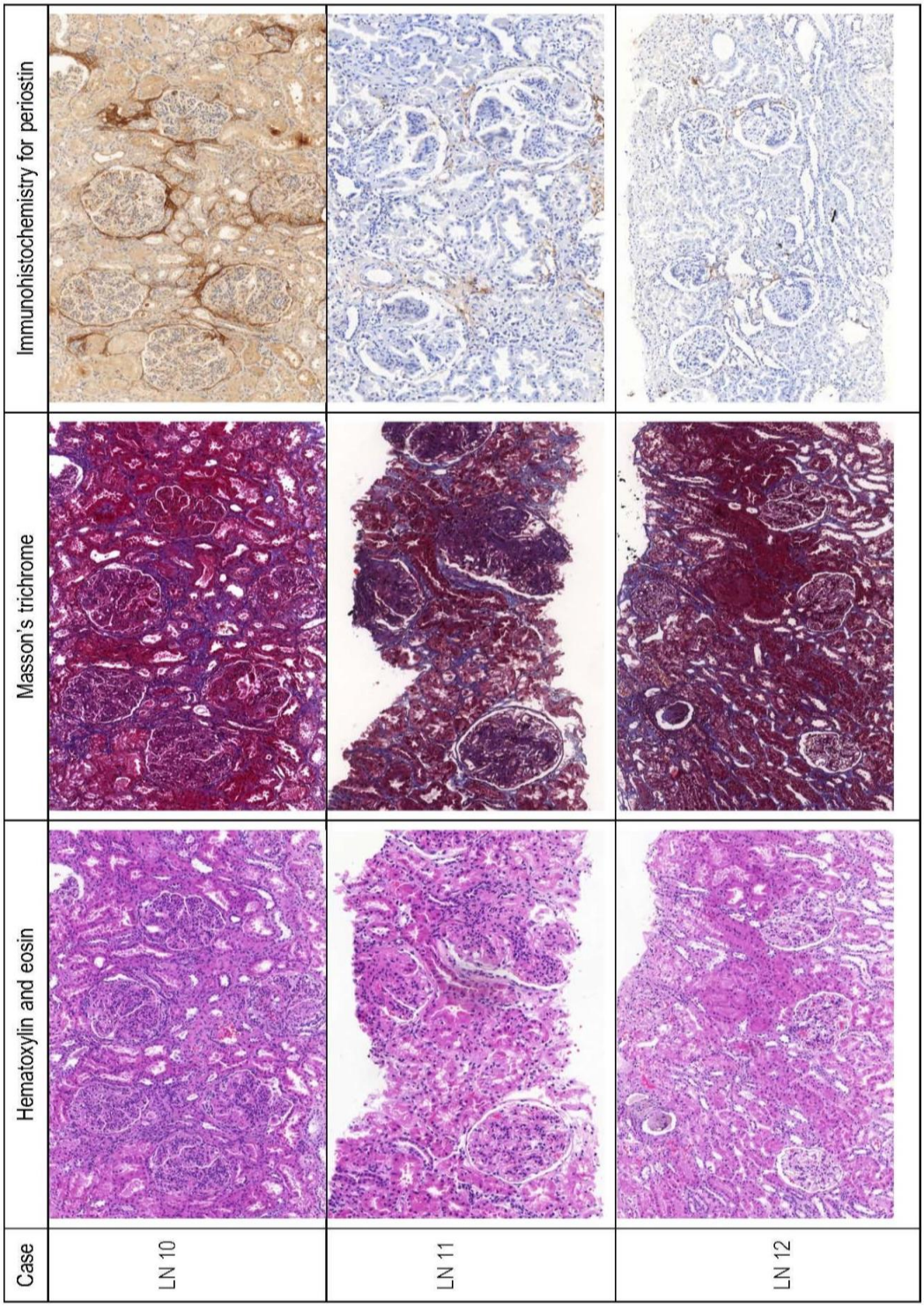
Control	Hematoxylin and eosin	Masson's trichrome	Immunohistochemistry for periostin
Control 1			
Control 2			

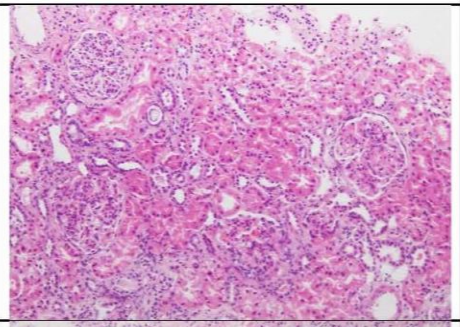
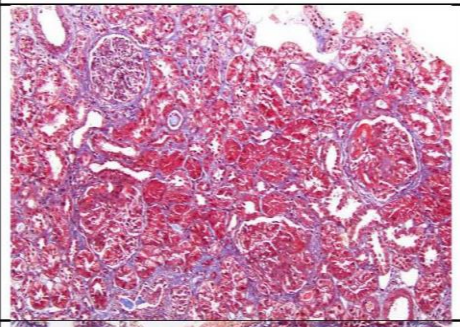
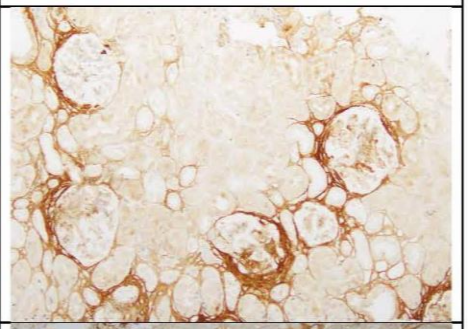
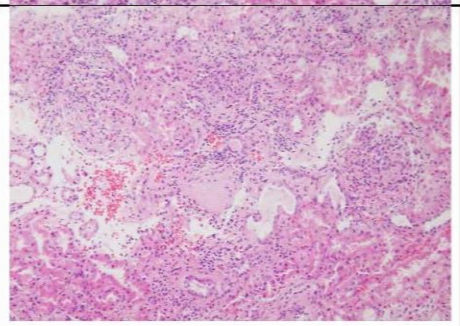
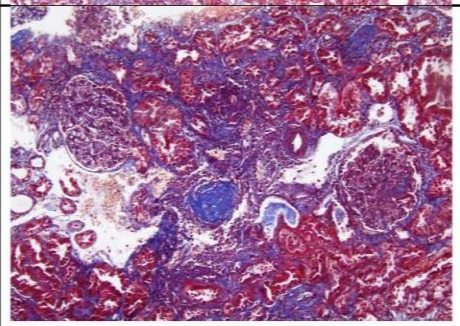
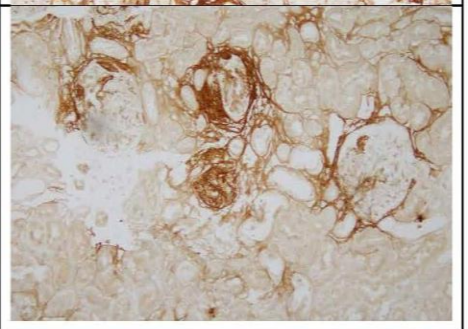
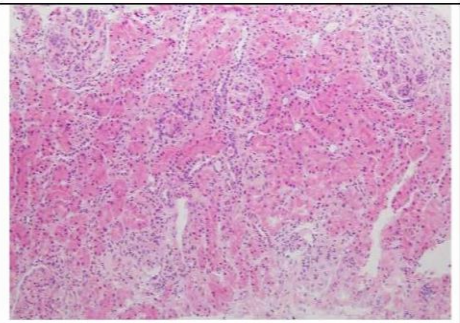
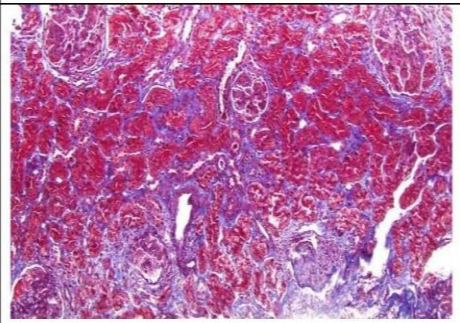
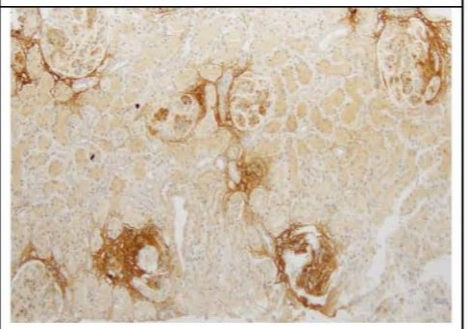

Control
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
Control 3
Control 4
Control 5

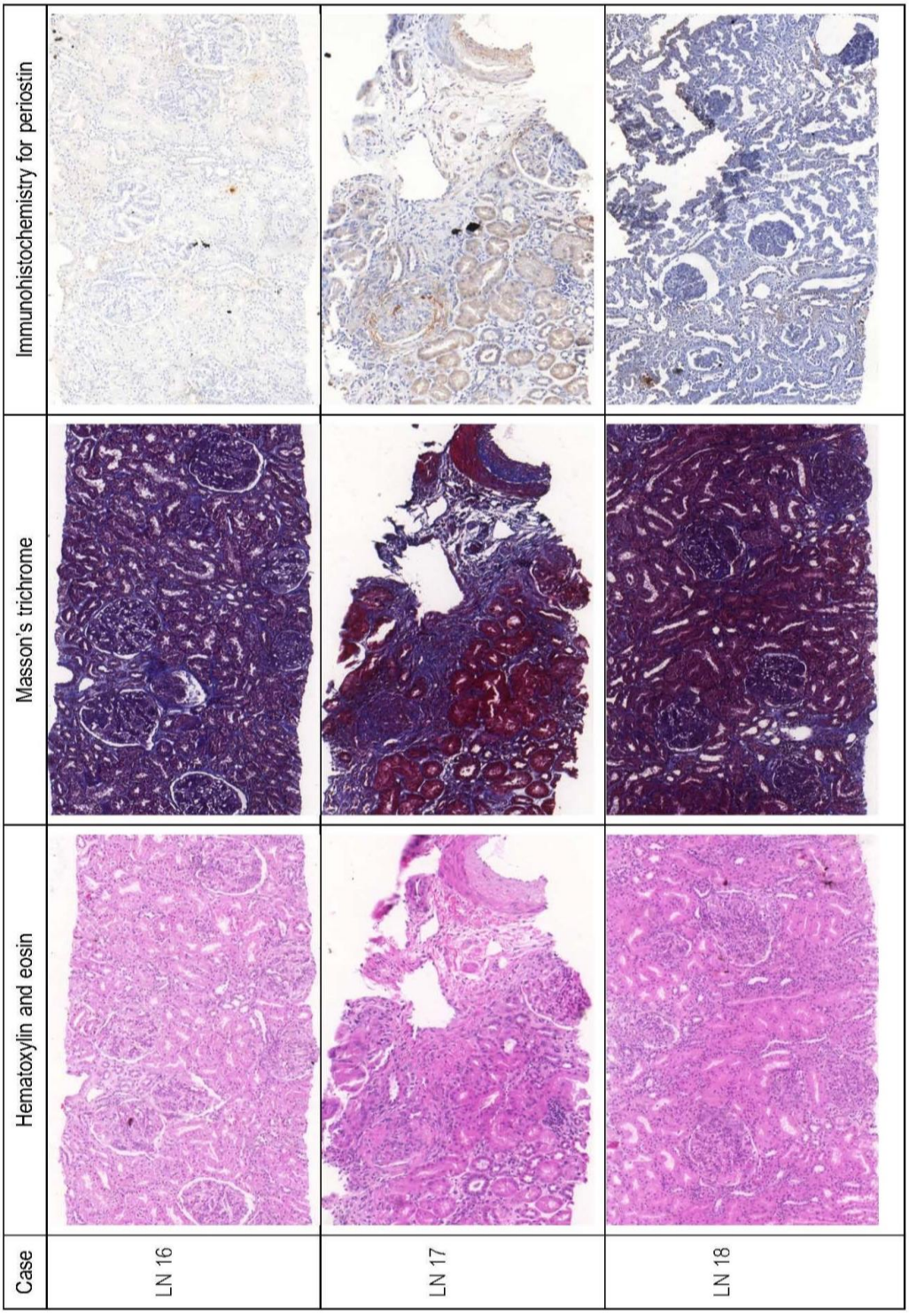

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 1
LN 2
LN 3

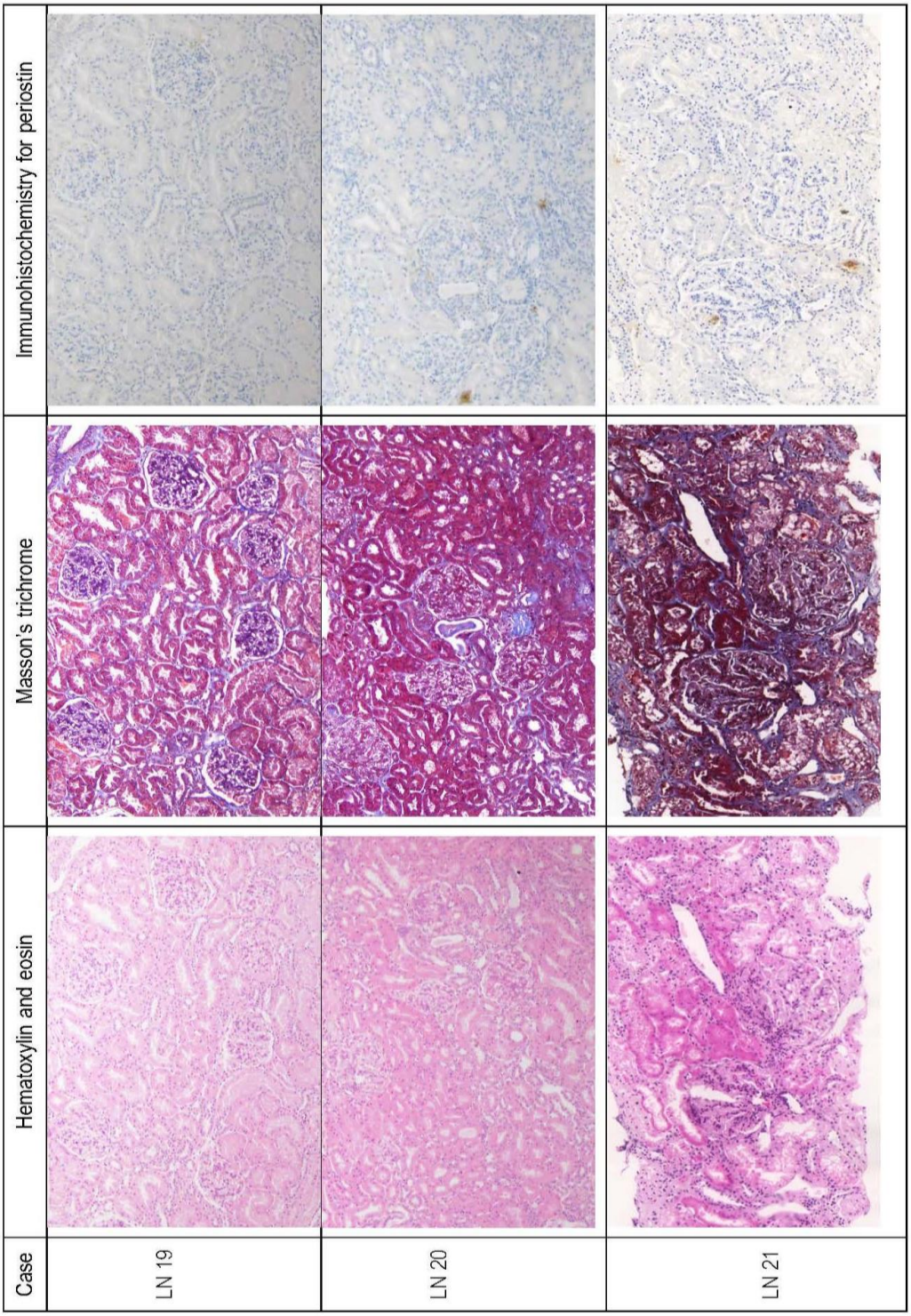
Case	Hematoxylin and eosin	Masson's trichrome	Immunohistochemistry for periostin
LN 4			
LN 5			
LN 6			

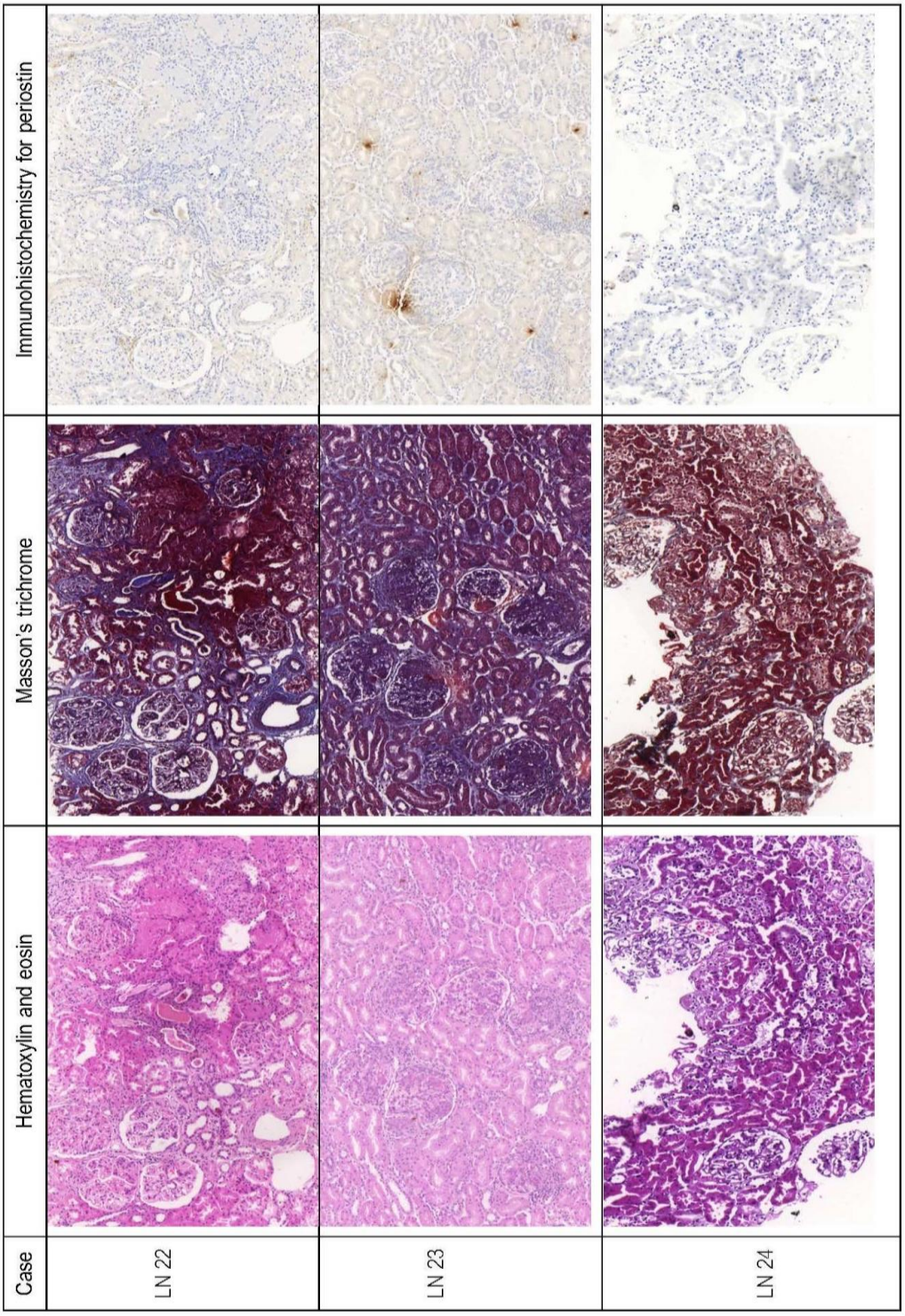
Case	Hematoxylin and eosin	Masson's trichrome	Immunohistochemistry for periostin
LN 7			
LN 8			
LN 9			

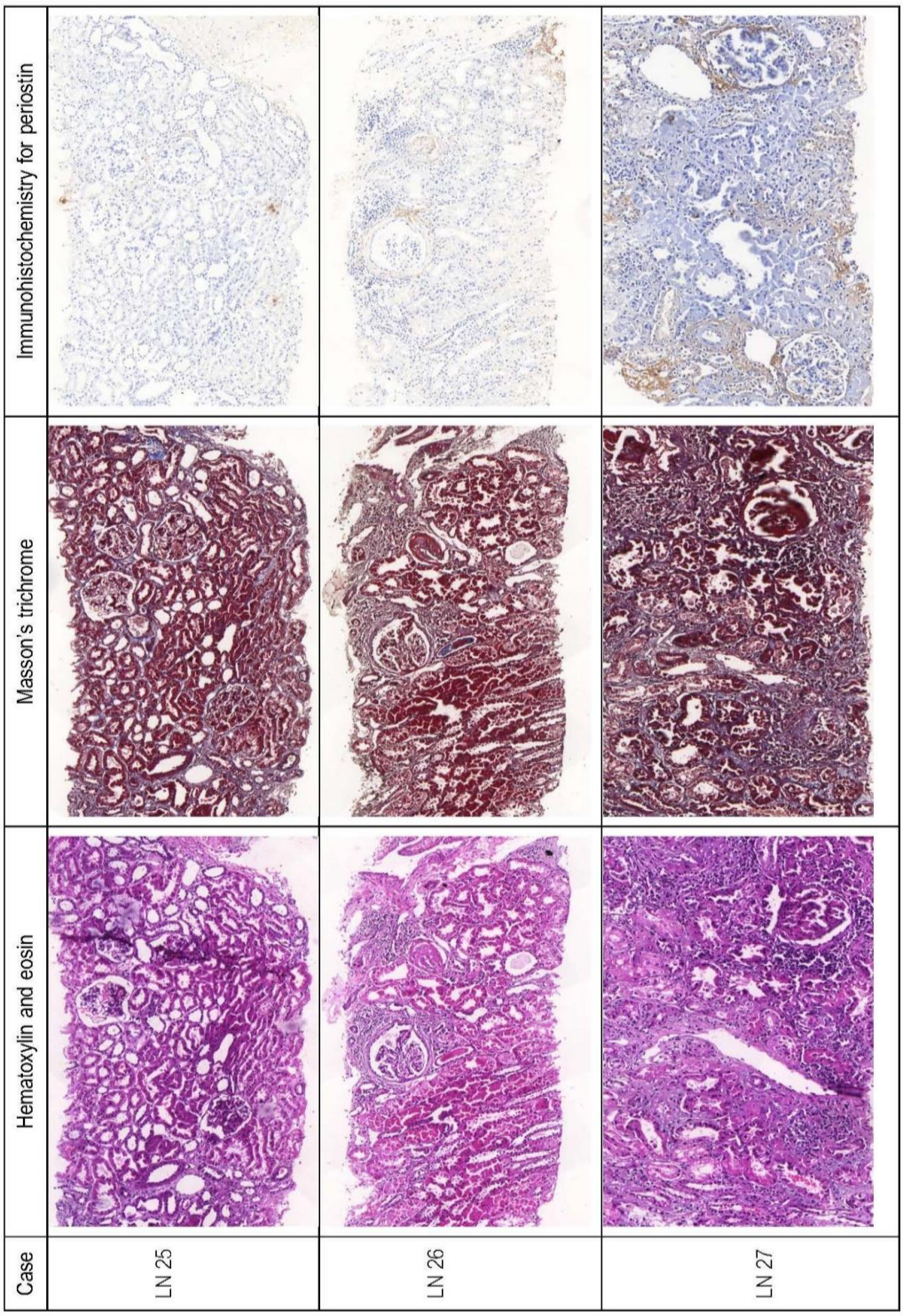

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 10
LN 11
LN 12

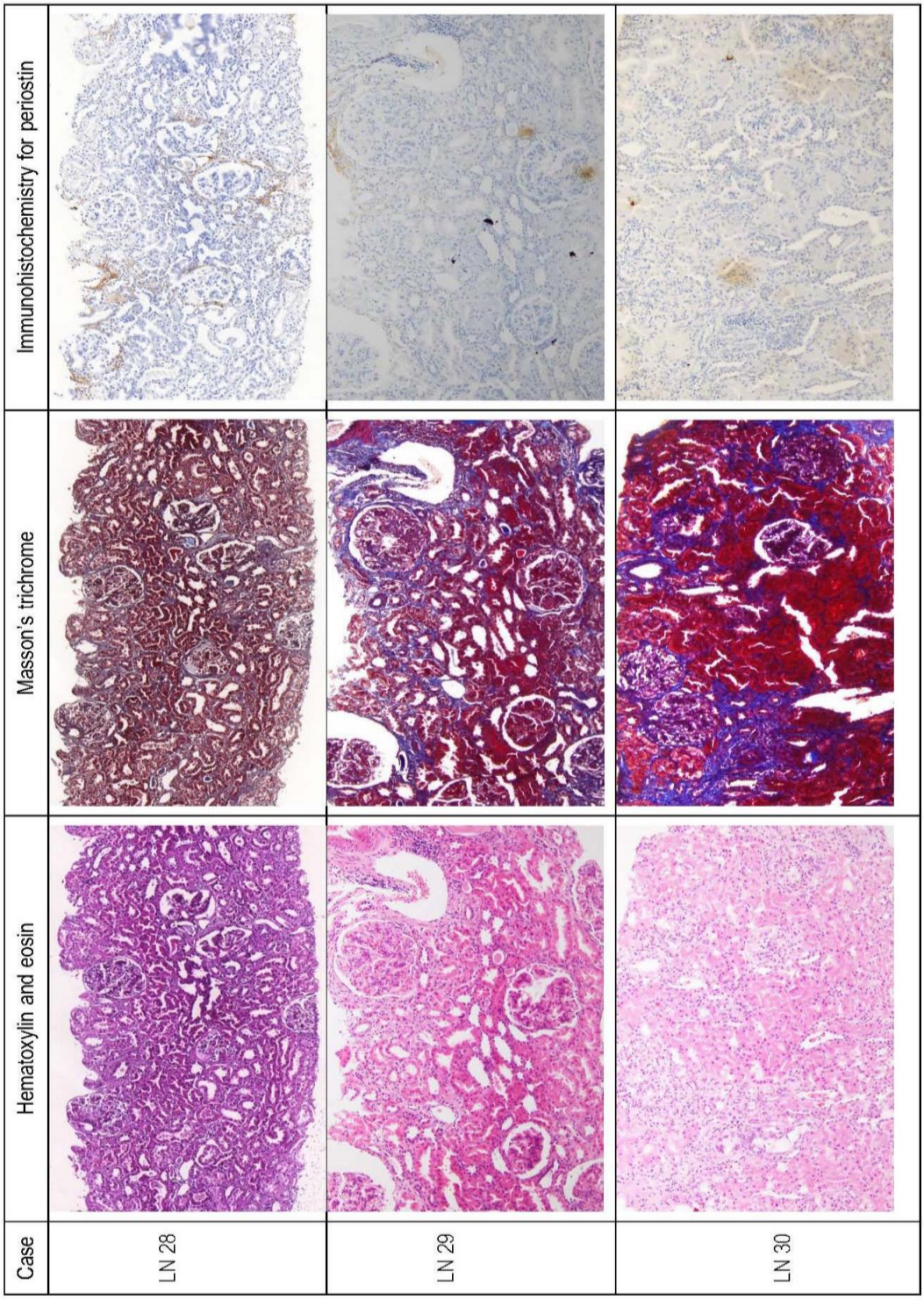
Case	Hematoxylin and eosin	Masson's trichrome	Immunohistochemistry for periostin
LN 13			
LN 14			
LN 15			

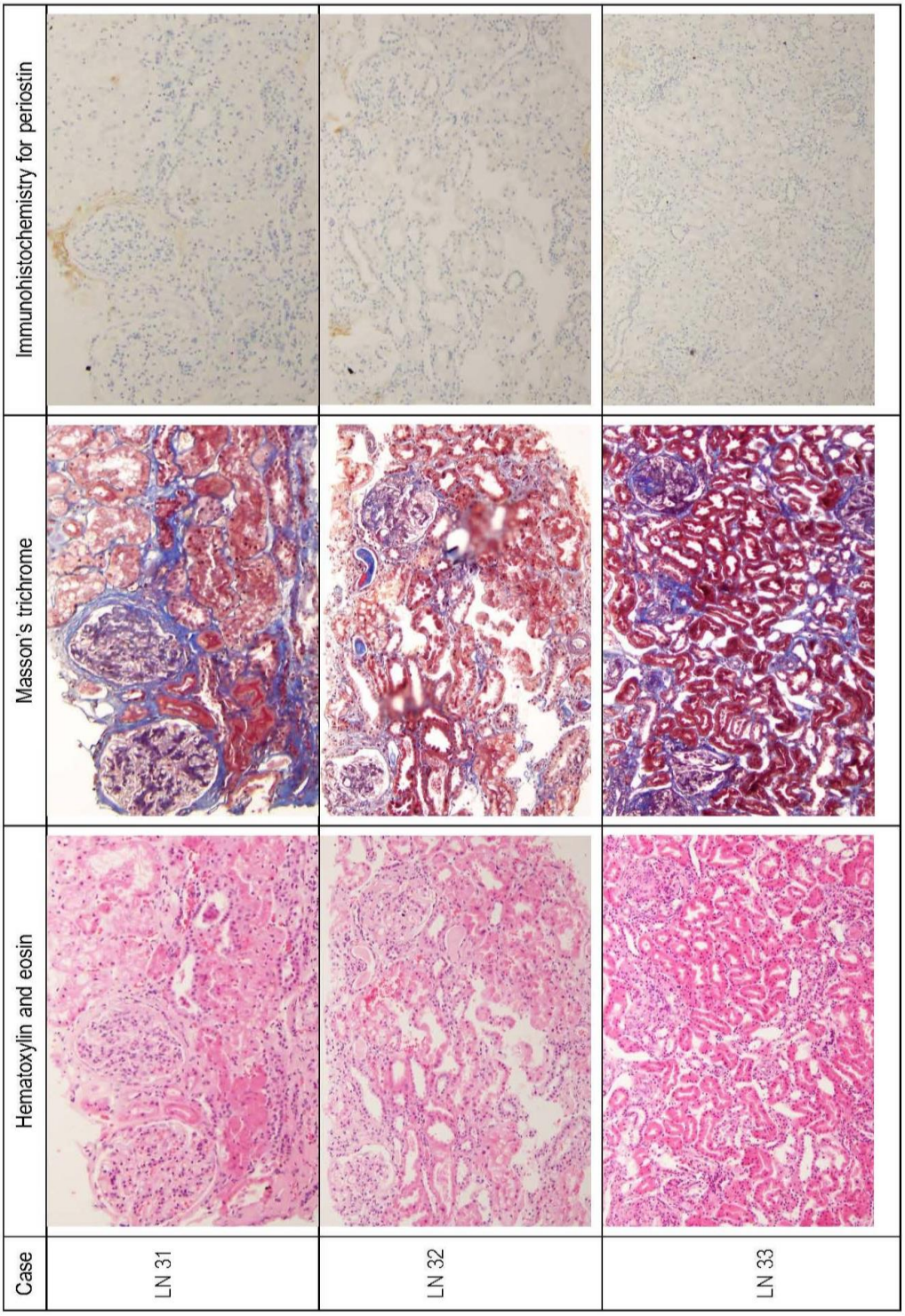

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 16
LN 17
LN 18

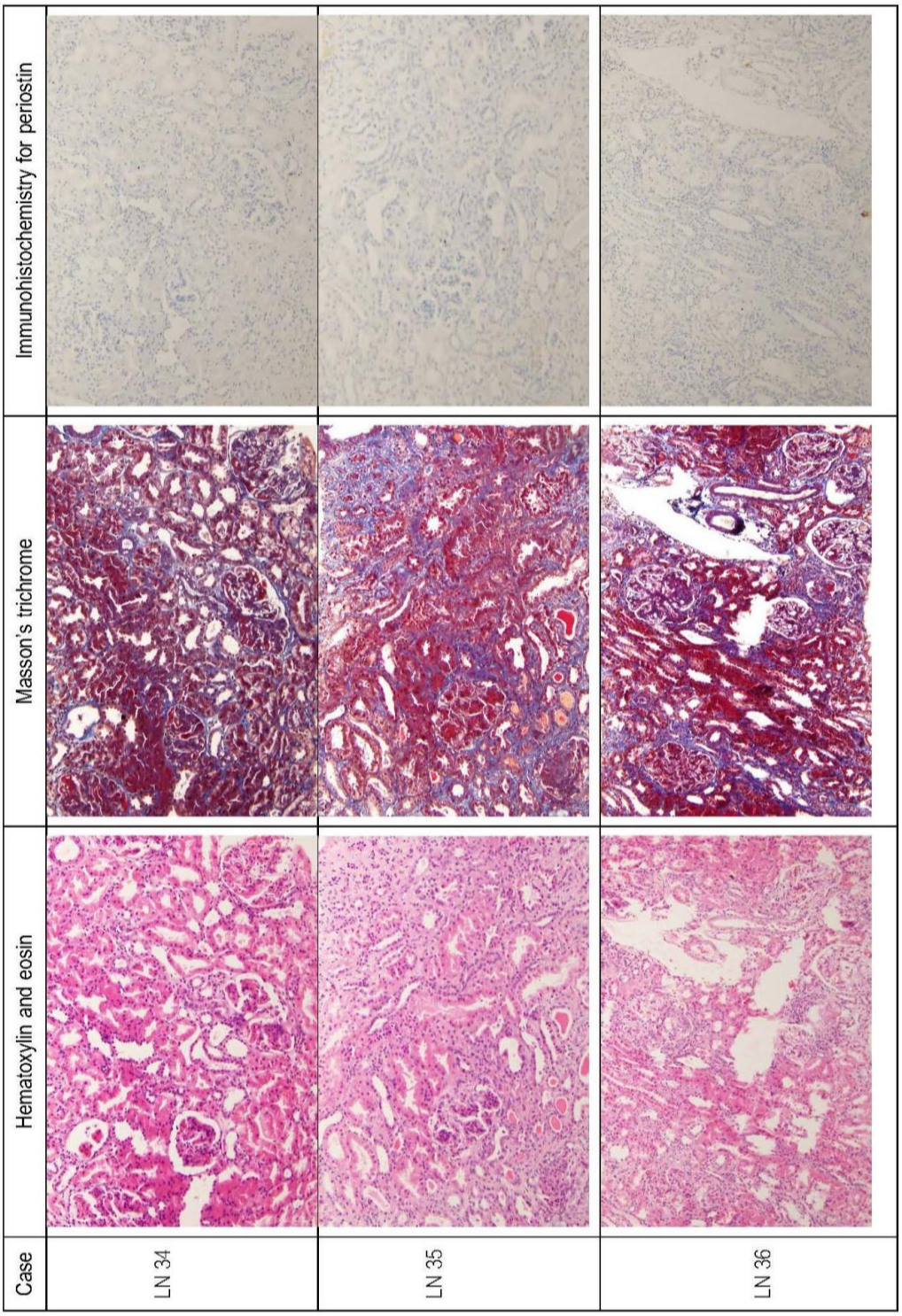

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 19
LN 20
LN 21

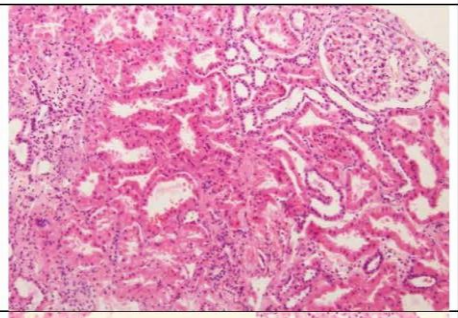
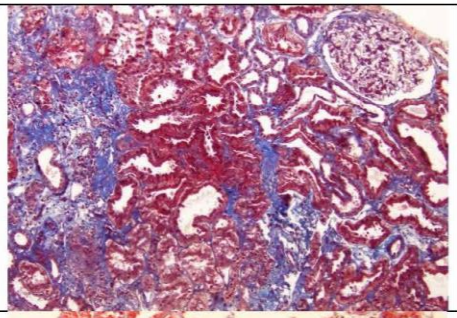
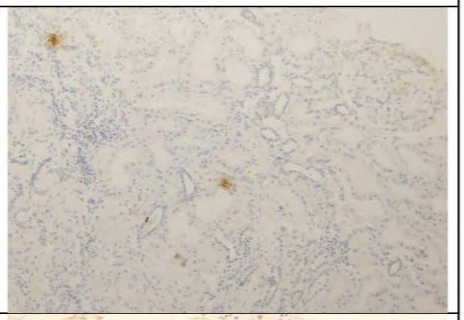
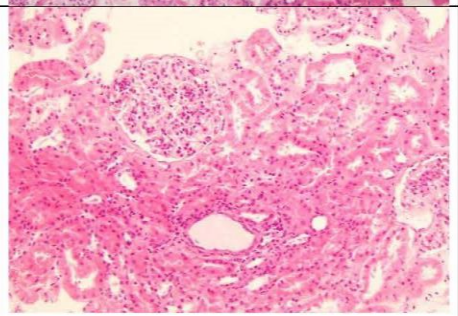
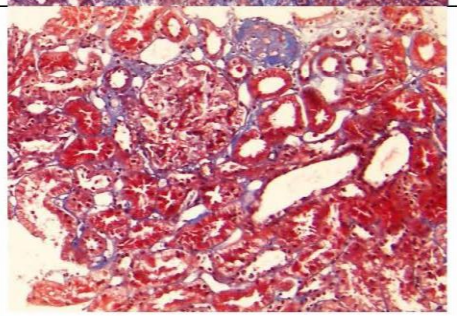
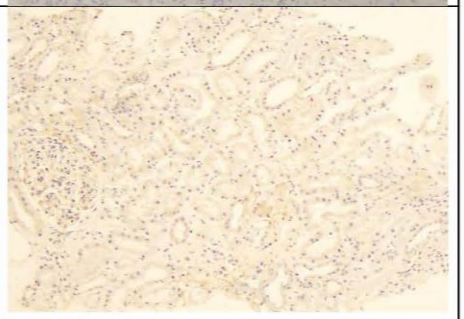
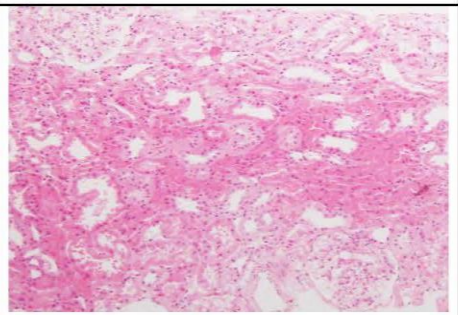
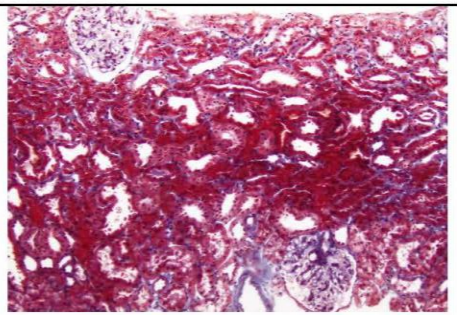
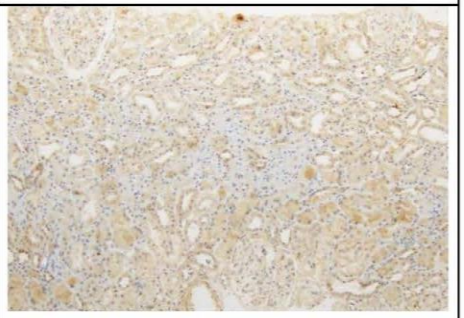

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 22
LN 23
LN 24

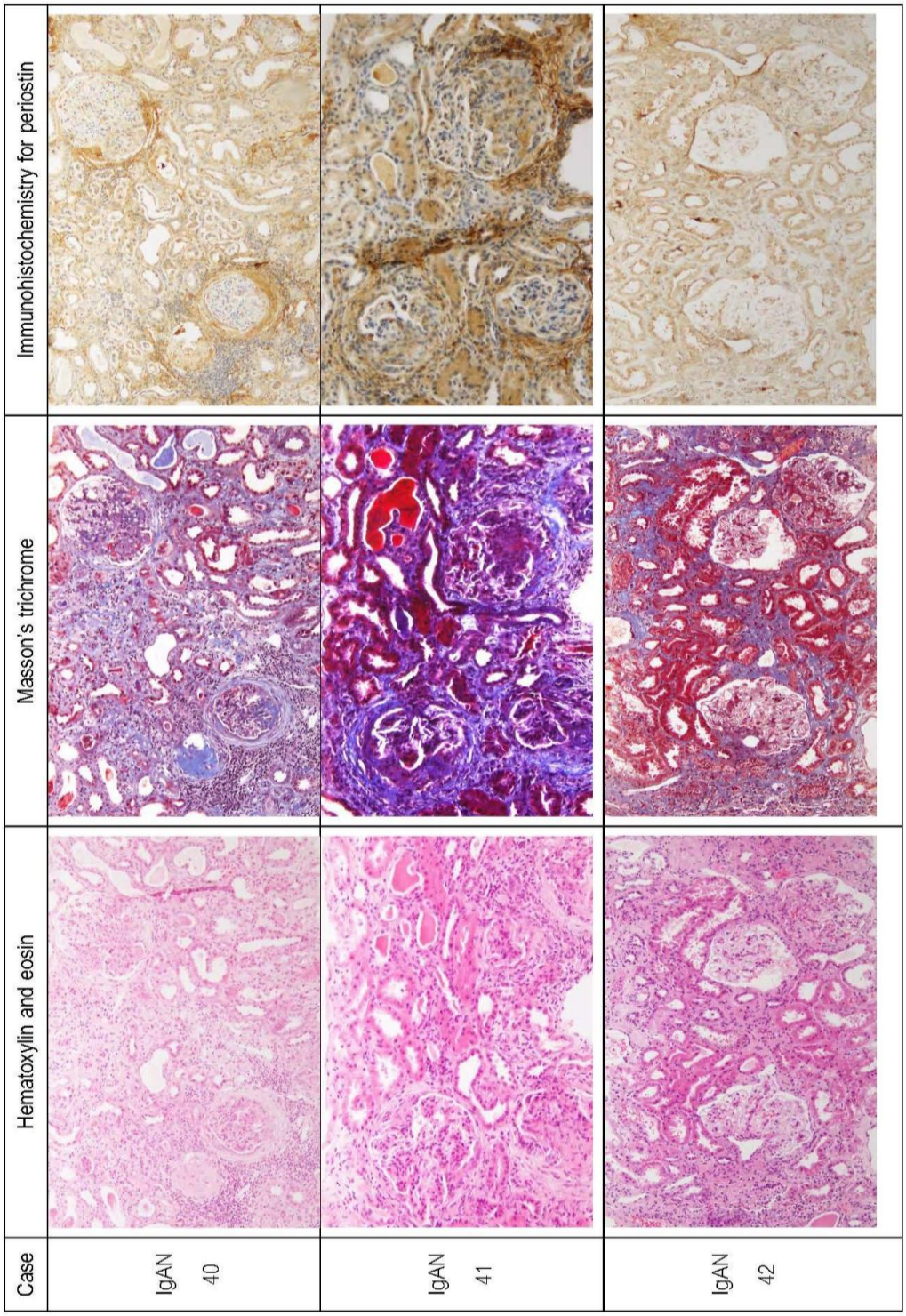

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 25
LN 26
LN 27

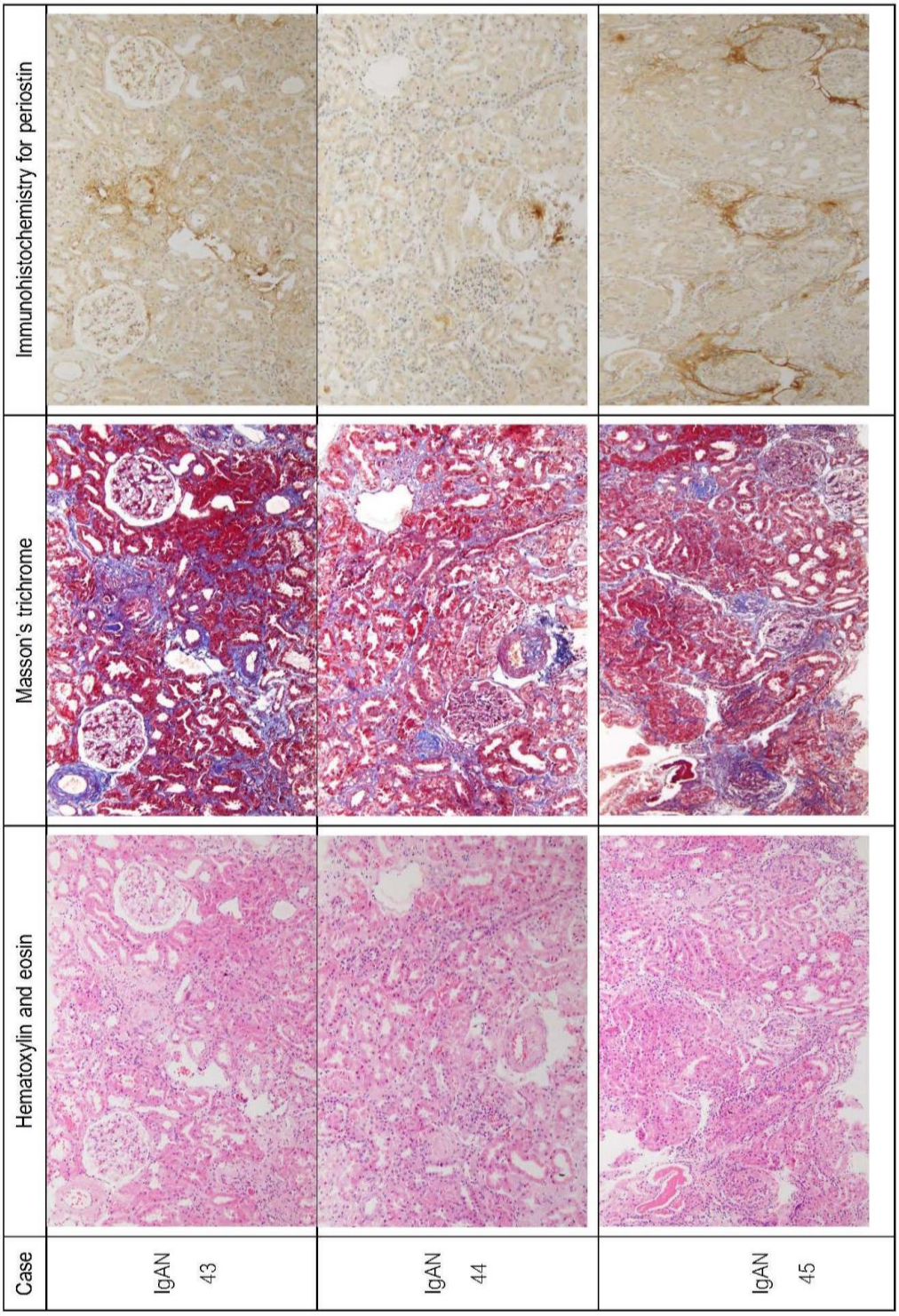

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 28
LN 29
LN 30

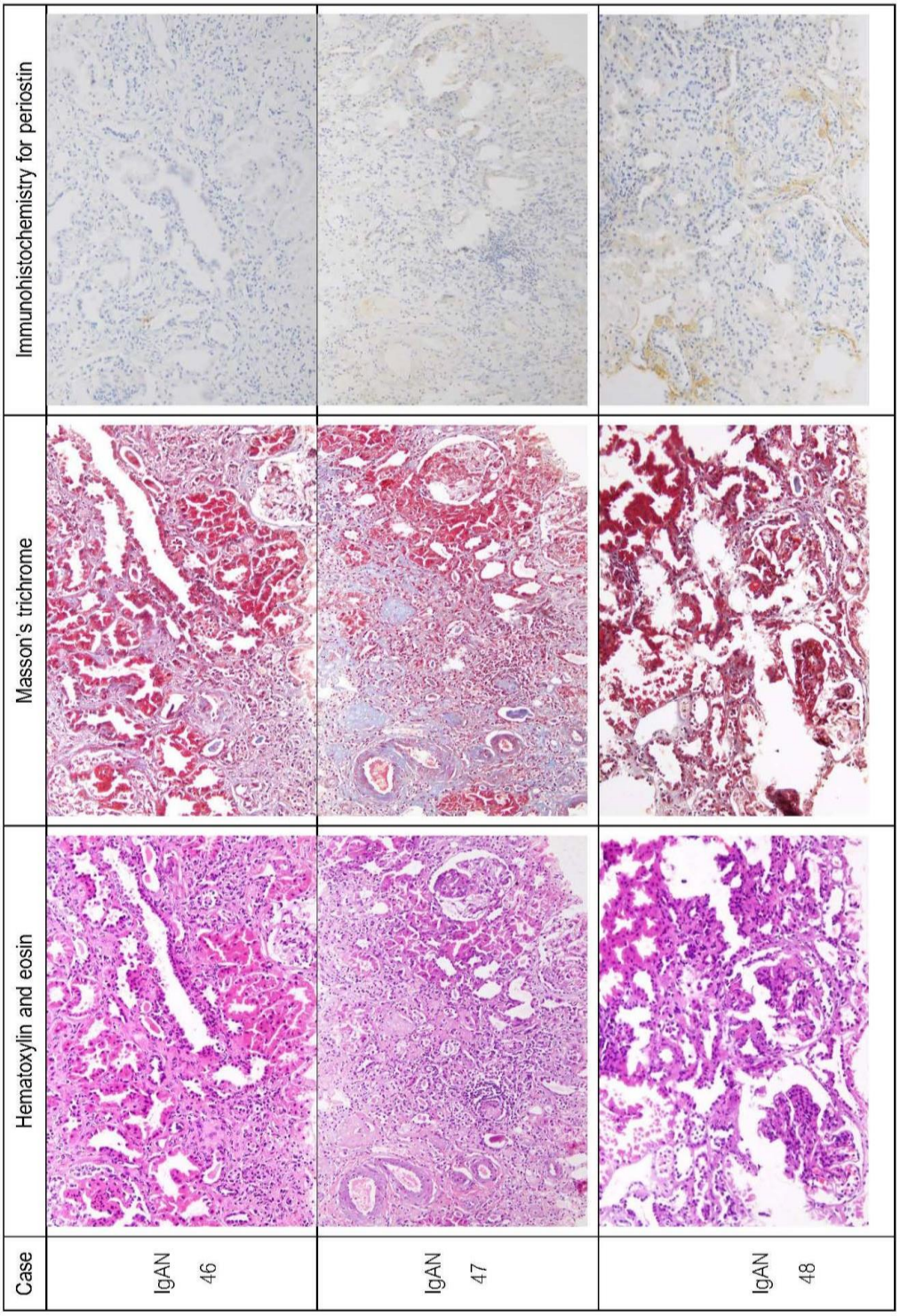

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 31
LN 32
LN 33

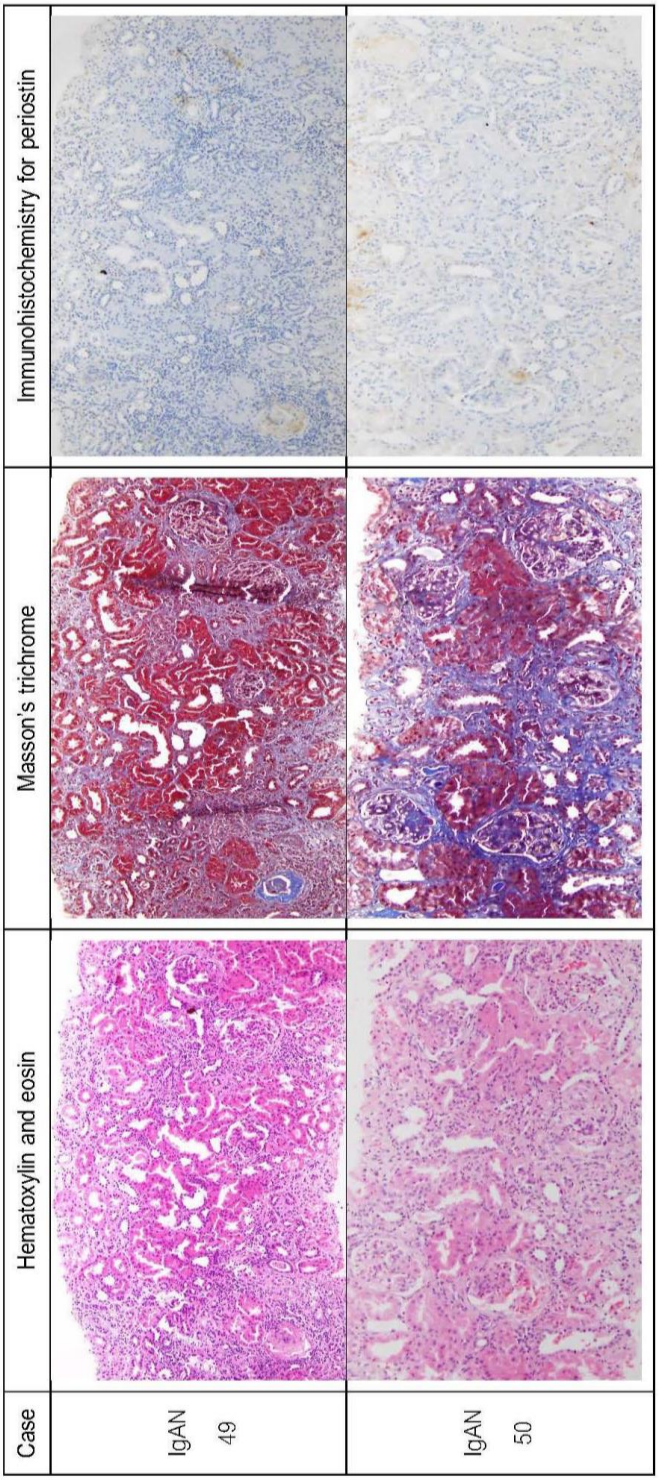

Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
LN 34
LN 35
LN 36

Case	Hematoxylin and eosin	Masson's trichrome	Immunohistochemistry for periostin
LN 37			
IgAN 38			
IgAN 39			


Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
IgAN 40
IgAN 41
IgAN 42


Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
IgAN 43
IgAN 44
IgAN 45


Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
IgAN 46
IgAN 47
IgAN 48


Case
Hematoxylin and eosin
Masson's trichrome
Immunohistochemistry for periostin
IgAN 49
IgAN 50

VITA

Ms. Peepattra Wantanasiri was born on November 13th, 1984 in Bangkok, Thailand. In 2008, she graduated with a Bachelor of Science in Pharmacy from Chulalongkorn University, Bangkok, Thailand (1st Class Honors). In 2008 to 2010, she worked as a pharmacist in the Department of Medical Supply, King Chulalongkorn Memorial Hospital, Bangkok, Thailand. In 2010, she was a Ph.D. student in Pharmaceutical Care (International Program) at Chulalongkorn University, Bangkok, Thailand and received a grant from the Thailand Research Fund through the Royal Golden Jubilee Ph.D. Program (grant no. PHD/0077/2552) to Peepattra Wantanasiri and Pornanong Aramwit.