# การศึกษาการแสดงออกของโปรตีนในปัสสาวะของผู้ป่วยไตอักเสบลูปัส

นางสาวภูริชญา สมภาร

วิทยานิพนธ์นี้เป็นส่วนหนึ่งของการศึกษาตามหลักสูตรปริญญา
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# Urinary proteomics of Lupus nephritis

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**A Dissertation Submitted in Partial Fulfillment of the Requirement**
**for the Degree of Doctor of Philosophy Program in Biomedical Sciences**
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ภูริชญา สมภาร: การศึกษาการแสดงออกของโปรตีนในปัสสาวะของผู้ป่วยไตอักเสบลูปัส (Urinary proteomics of Lupus nephritis) อ.ที่ปรึกษาวิทยานิพนธ์หลัก: รศ.นพ.ยิ่งยศ อวิหิงสานนท์, อ.ที่ปรึกษาวิทยานิพนธ์ร่วม: รศ.ดร.พญ ณัฏฐิยา หิรัญกาจณ์, รศ.ดร.นพ. วีรพันธุ์ โชควิฑูรกิจ, 80 หน้า.

โรคลูปัสเป็นโรคไตที่สำคัญที่นำไปสู่ภาวะไตเรื้อรังที่พบได้บ่อยในกลุ่มของชาวเอเชีย ปัจจุบันการวินิจฉัยและพยากรณ์โรคในผู้ป่วยไตอักเสบจากโรคลูปุสทำได้โดยการตัดชิ้นเนื้อไตเพื่อตรวจหาพยาธิสภาพ แต่วิธีนี้มีข้อเสียคือมีความยุ่งยากและผู้ป่วยมีความเสี่ยงของภาวะแทรกซ้อนจากการตัดเนื้อเยื่อไตได้ ดังนั้น การศึกษานี้จึงมีวัตถุประสงค์เพื่อค้นหาเครื่องหมายทางชีวภาพ (biomarkers) เพื่อใช้เป็นดัชนีบ่งชี้ลักษณะของผู้ป่วยที่มีอาการกำเริบแยกออกจากผู้ป่วยที่มีอาการสงบของผู้ป่วยไตอักเสบลูปัส นอกจากนี้ยังได้มีการศึกษาหาโปรตีนที่ตอบสนองต่อการรักษาด้วยโดยใช้เทคนิค 2-dimensional gel electrophoresis และ LC-MS/MS ผลการศึกษาพบว่า โปรตีน 13 ชนิดที่แตกต่างกันระหว่างของผู้ป่วยที่มีอาการกำเริบและกลุ่มผู้ป่วยที่มีอาการสงบจากการตรวจสอบด้วยวิธี enzyme-linked immunosorbent assay พบว่าโปรตีน 2 ชนิด คือ zinc-$\alpha$2-glycoprotein (ZA2G) และ Prostagladin $H_2$ D-isomerase (PGDS) เพิ่มขึ้นในกลุ่มตัวอย่างคนไข้ โปรตีน ZA2G มีการหลั่งออกมาในปัสสาวะของคนไข้ที่มีอาการกำเริบมากกว่าอาการสงบและมีปริมาณการหลั่งในปริมาณที่ใกล้เคียงกันกับกลุ่มคนไข้ที่มีการรั่วของโปรตีนในปัสสาวะของคนที่ไม่มีการอักเสบของไต สำหรับโปรตีน PGDS พบว่ามีการหลั่งในปัสสาวะของคนไข้ที่มีอาการกำเริบมากกว่าอาการสงบและกลุ่มคนไข้โปรตีนรั่วในปัสสาวะอย่างมีนัยสำคัญทางสถิติ ดังนั้นจึงมีความเป็นไปได้ว่าโปรตีน PGDS อาจสามารถเป็นโปรตีนเครื่องหมายเพื่อใช้ในพยากรณ์การกำเริบของผู้ป่วยไตอักเสบลูปัสได้ นอกจากนั้นยังได้หากลุ่มโปรตีนที่แตกต่างกันระหว่างในขณะที่กลุ่มผู้ป่วยที่ตอบสนองและไม่ตอบสนองต่อการรักษา พบว่ามีโปรตีน7 ชนิดที่แตกต่างกันอย่างมีนัยสำคัญทางสถิติ โดยที่โปรตีน CD59 และ A1M เป็นโปรตีน ที่มีหน้าที่ในการเป็น immunoprotective นอกจากนั้นยังพบว่าโปรตีนทั้งสองชนิดมีการแสดงออกในกลุ่มคนไข้ที่ตอบสนองต่อการรักษาแตกต่างกับกลุ่มคนไข้ที่ไม่ตอบสนองต่อการรักษาอย่างมีนัยสำคัญ ซึ่งการค้นพบนี้เป็นข้อมูลที่สำคัญที่จะนำไปพัฒนาการศึกษาต่อไปในอนาคตในกลุ่มคนไข้ที่มีจำนวนมากขึ้น เพื่อใช้เป็นตัวบ่งชี้ในการพยากรณ์ผลสำเร็จของการรักษาได้

สาขา ชีวเวชศาสตร์ ลายมือชื่อนิสิต.....

ปีการศึกษา 2554 ลายมือ อ.ที่ปรึกษาวิทยานิพนธ์หลัก.....

ลายมือ อ.ที่ปรึกษาวิทยานิพนธ์ร่วม.....

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# # 4989685620: MAJOR BIOMEDICAL SCIENCE
KEYWORDS: LUPUS NEPHRITI/KIDNEY/ZINC-α2-GLYCOPROTEIN/
PROSTAGLADIN $H_2$ -D ISOMERASE/CD59 GLYCOPROTEIN/UIRNE

POORICHAYA SOMPARN: URINARY PROTEOMICS OF LUPUS NEPHRITIS. ADVISOR: ASSOC.PROF. YINGYOS AVIHINGSANON, M.D., CO-ADVISOR: ASSOC.PROF. NATTIYA HIRANKARN, M.D., ASSOC.PROF. WEERAPAN KHOVIDHUNKIT, M.D., 80 pp

Lupus nephritis (LN) is one of the common causes of chronic kidney disease, especially in Asian ethnic. The kidney biopsy score is currently the most important diagnostic and severity-determined biomarker. Inevitably, the non-invasive biomarker is interesting for diagnosis and prognosis of LN due to the risk of post-renal biopsy complications. Urine proteomics are the current interesting tools to enhance discovery of protein urinary biomarkers. In this study, we used 2-dimensional gel electrophoresis (2-DE) and identified the spot difference by electrospray/ liquid chromatography/ mass spectrometry technique (ESI/LC/MS) between urine of active and inactive LN patients. Sixteen proteins were quantitatively different. Zn-alpha2-glycoprotein (ZA2G) and prostaglandin $H_2$ D-isomerase (PGDS) were validated by enzyme-linked immunosorbent assay in a large patient sample set. Urinary ZA2G was increased in both active LN and other glomerular diseases whereas PGDS was increased specifically in active LN. Urinary PGDS might be a good diagnostic and prognostic biomarker for active LN. Moveover, we have studied in patient after treatment with immunosuppressive medicine. Seven proteins were differentially expressed between groups. CD59 and A1M play an immunoprotective role. Further validation in a larger sample size is needed.

Field of Study: Biomedical Science Student's Signature……
Academic Year: 2011 Advisor's Signature……...
Co-advisor's Signature……..
Co-advisor's Signature……..

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| | |
|---|---|
| 2-DE | Two dimensional gel electrophoresis |
| A1M | Alpha-1-microglobulin |
| ACN | Acetronitrile |
| °c | Degree Celsius |
| CE/MS | Capillary electrophoresis coupled to mass spectrometry |
| DI | Deionized water |
| dsDNA | Double–standed deoxyribonucleic acid |
| DNA | Deoxyribonucleic acid |
| DTT | Dithiothreitol |
| ESI | Electrospray ionization |
| ESRD | End-stage renal disease |
| FTICR | Fourier transform ion cyclotron resonance |
| GFR | Glomerular filtration rate |
| GBM | Glomerular basement membrane |
| HSPG | Heparin sulfate proteoglycan |
| IAA | Iodoacetamide |
| IC | Immune complex |
| IEF | Isoelectric focusing |
| IgG | Immunoglobulin G |
| IT | ion-trap |
| kDa | Kilo Dalton |
| LC/MS | liquid chromatography coupled to mass spectrometry |
| LN | Lupus nephritis |

| | |
|---|---|
| MALDI | Matrix-assisted laser desorption/ionization |
| Ml | Milliliter |
| mM | Millimolar |
| MW | Molecular weight |
| MS | Mass spectrometry |
| PGDS | Prostagladin $H_2$ D isomerase |
| pI | Isoelectric point |
| RBC | Red blood cell |
| SDS-PAGE | Sodium dodecyl sulfate-polyacrylamide gel electrophoresis |
| SELDI/TOF | Surface-enhanced laser desortion/ionization coupled to mass spectrometry |
| SLE | Systemic lupus erythematosus |
| TOF | Time-of-flight |
| WBC | White blood cell |
| WHO | The World Health Organization |
| µl | Microlitter |
| µg | Microgram |
| UPCI | Urine protein creatinine index |
| ZA2G | Zinc-alpha2-glycoprotein |

# CHAPER I

# INTRODUCTION

Lupus nephritis (LN) is the second leading cause of glomerular diseases in Asia. It is the most common and serious complication of systemic lupus erythematosus (SLE) (Hill, Delahousse, 2002). One-third of patients dies or reaches end-stage kidney disease. Most of patients present with LN suffer a relapse during treatment. The use of immunosuppressive drug treatment which is necessary to control the active disease can lead to severe side effects for the patients, among others susceptibility towards infections ( Avihingsanon and Hirankarn, 2010).

The understanding of the renal pathology is crucial to predict the prognosis of the patients. While patients with inactive and active LN have a good chance to maintain normal renal function, patients with class active LN frequently develop chronic renal failure (Avihingsanon and Hirankarn,2010). Currently, the diagnostic criteria for LN consist of parameters of renal function tests such as urine protein content, urine sediment of red and white blood cells as well as histologic evaluation following a renal biopsy (Mosley, Tam, 2006).

The renal function parameters do not necessarily correlate with the inflammatory activity. Their prognostic value is limited. Renal biopsy is the gold standard in assessment of renal disease and disease activity. However, renal biopsy is associated with many risks, e.g. severe bleeding. It would be of great help to have a noninvasive method of similar prognostic value. This non-invasive tool could help to monitor the disease and guide to treatment in case of a relapse.

Recently, 2DE is well known to discover the biomarker identification in many

diseases. The 2DE is a high-resolution technique that can be coupled with protein identification by mass spectrometry (Thongboonkerd, 2008). Urine biomarker discovery is an available source to visualize differential protein in renal disease. In the study, we used 2-DE gel to identify a differentially expressed protein in urine of patients with SLE that is divided into inactive VS active LN and response VS non-response to identify urine protein that can be used as novel biomarker for active LN and following to treatment.

**2. Research Questions**

1. Do proteins in urine differentially excreted between inactive and active LN?

2. Do proteins in urine differentially excreted between LN that response and nonresponse to treatment?

**3. Objectives**

1. To identify differentially excreted proteins in urine of inactive and active LN.

2. To identify differentially excreted proteins in urine of LN that response and nonresponse to treatment.

**4. Hypothesis**

There are differences in the expression of proteins in urine

# CHAPTER II

# LITERATURE REVIEW

Systemic Lupus Erythematosus (SLE) is a prototypic autoimmune diseases characterized by the production of autoantibodies that can affect any part of the body. The genetic and environmental factors were believed that cause of pathogenesis. The onset of SLE is typically between the ages of 15 and 40 years with 90% of these affected individuals being female. In the United States, a Caucasian female has an approximately 1 in 700 chance of developing SLE during her lifetime (Kimberly, 2001). Multi organs involvements in SLE are occur, especially vital organs, such as nervous system, heart, lungs, blood system and kidneys.

## 1. Lupus nephritis

Lupus nephritis is renal disease that common and serious manifestation of systemic lupus erythematosus (SLE) and one of the most complication in SLE, which involves kidneys including vascular, glomerular and tubulointerstitial lesions, and occurring in up to 60% of patients with SLE. It is one of the most leading causes of morbidity and mortality in systemic lupus erythematosus (SLE) (Lea, 2002).The important issue of patients with lupus nephritis is asymptomatic urinary abnormalities to rapidly progressive renal failure leading to end-stage renal disease**.** The progression to LN in SLE is thought to be dependent on the loss of self-tolerance and the formation of autoantibodies that deposit in the kidney to induce nephritis.

### 1.1 Etiology of Lupus Nephritis

Abnormal apoptosis and inadequate removal of apoptotic cells and nuclear remnants might contribute to autoimmunity by causing prolonged exposure of the immune system to nuclear and cell membrane components (Stuart and Hughes, 2002). Recently studies have described specific genetic linkage to the progression of renal disease in SLE among certain ethnic groups, including European American and African American populations, some of which may determine the severity of the glomerular disease (Lea, 2002).

### 1.2 Pathogenesis and Mediation of Disease

Combination between environmental factors and genetic abnormalities of the immune system were determined SLE is an autoimmune disease with a complex pathogenesis. The immune dysregulation in lupus nephritis is characterized by polyclonal B-cell activation, which induced by cognate autoreactive helper T-cells, and the formation of autoreactive antibodies directed against nuclear antigen and other self-antigen, so-call autoantigen (Hahn, 1998). High-affinity, IgG anti-double-stranded DNA (anti-dsDNA) antibodies and glomerular immunoglobulin deposits are correlated with lupus nephritis. In addition, excretion of immunoglobulin from glomeruli revealed enrichment for anti-dsDNA antibodies. Therefore, it has been postulated that anti-dsDNA autoantibodies are nephritogenic in lupus nephritis. There are 2 mechanisms that can be explained binding of anti-double-stranded DNA (anti-dsDNA) autoantibodies to glomerular basement membrane (GBM) in lupus nephritis: (1) direct cross-reactive binding to intrinsic glomerular antigens; (2) nucleosome-

mediated binding to heparin sulfate in the GBM (Figure 1) (van Bavel, van der Vlag, 2007)

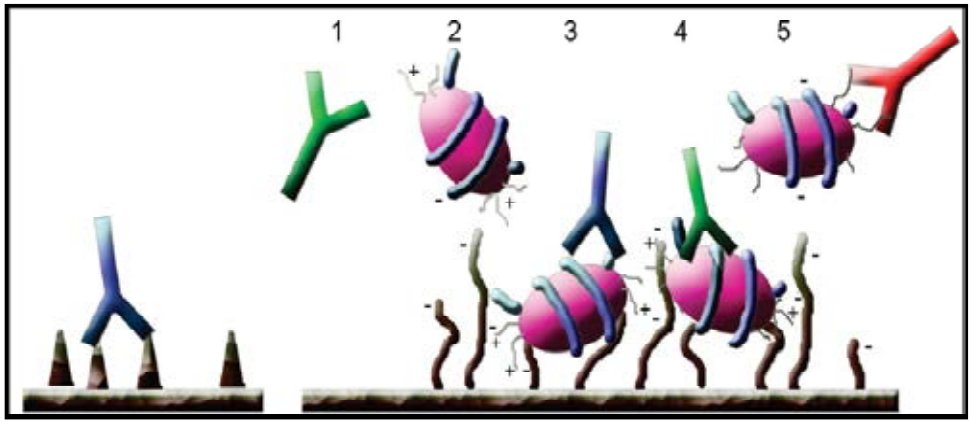


**Figure 1.** The two hypotheses for the glomerular binding of autoantibodies in lupus nephritis. From left to right: direct binding of cross-reactive autoantibodies to non-nucleosomal glomerular antigens; nucleosome-mediated binding of complexed autoantibodies to heparan sulfate (HS). The nucleosome-mediated binding does not occur with (1) non-complexed anti-nuclear antibodies or (2) free nucleosomes. Binding of (3) anti-dsDNA (blue) or (4) anti-nucleosome antibodies (green) will decrease the density of negative charges of the nucleosome. This will enhance binding of the complex to the negatively charged HS and lead to nucleosome-mediated binding. In contrast to this, binding of (5) anti-histone antibodies (red) to the nucleosome will decrease the amount of positive charges, which reduces the capacity to bind to HS in the glomerular basement membrane and preventing deposition of nucleosome/immune complexes.

### 1.3 Renal Manifestations of Lupus Nephritis

Proteinuria is the most feature of renal lupus as shown in Table 1. This feature is presented in almost every patient and commonly leading to the nephrotic syndrome.

The patients also have a microscopic hematuria in urine, but never in isolation; a macroscopic hematuria is rare. Moreover, Some patient have hypertension but is not overall more common in those with nephritis. In addtiton, those with more severe nephritis are more commonly hypertensive. About half will have a feature a reduced GFR, and infrequent patients present with acute renal failure. Renal tubular function is disturbed, which is not surprising in view of the finding of both immune aggregates in tubular basement membranes and the presence of interstitial nephritis (see below). Urinary excretion of light chains and β2-microglobulin are both increased in a high proportion of patients. Recently, hyperkalemic renal tubular acidosis has been emphasized as a manifestation of lupus (Cameron, 1999).

**Table 1.** Clinical features of patients with lupus nephritis

| Feature | % of Those with Nephritis |
|---|---|
| Proteinuria | 100 |
| Nephrotic syndrome | 45 to 65 |
| Granular casts | 30 |
| Red cell casts | 10 |
| Microscopic hematuria | 80 |
| Macroscopic hematuria | 1 to 2 |
| Reduced renal function | 40 to 80 |
| Rapidly declining renal function | 30 |
| Acute renal failure | 1 to 2 |
| Hypertension | 15 to 50 |
| Hyperkalemia | 15 |
| Tubular abnormalities | 60 to 80 |

Although, Some lupus nephritis patients with no clinical evidence of renal involvement (no proteinuria, normal urine microscopy, normal renal function) showed active histological change on renal biopsy specimens. This known as “silent lupus nephritis” (4). Recently, the investigators found significant renal involvement (Class III, IV, or V LN) in SLE patients with < 1000 mg proteinuria with or without hematuria. These findings suggest that biopsy be strongly considered in this patient population (Christopher-Stine, Siedner, 2007).

### 1.4 The Classification of Lupus Nephritis

Based on various experimental models of autoimmune and immune complex disease in the kidney and observations in human renal biopsies, the classification of the various patterns of renal injury in SLE can be divided into three groups, including mesangial, endothelial and epithelial pattern (Weening, D'Agati, 2004).

The first World Health Organization (WHO) classification was formulated by Pirani and Pollak in Buffalo, New York in 1974 and was first used in publications in 1975 and 1978 (Weening, D'Agati, 2004). However, it is still doubtful for the value of the index and its reproducibility. In 1982, the WHO classification was modified by the International Study of Kidney Disease in Children. Diffuse proliferative lesion (type IV) is the most common and the most severe form of LN. Mesangial (type II) and membranous (type V) types are mild form and have relatively normal renal function. Focal proliferative type (type III) has quite variable clinical features. Progressive renal dysfunction is uncommon in type III with less than 25 percent of glomerular involvement (Banfi, Mazzucco, 1985).

Several studies have emphasized the usefulness of semiquantitative analysis to assess the activity and chronicity of nephritis (Banfi, Mazzucco, 1985, Austin, Boumpas, 1994). The maximum of activity score is 24 and the maximum of chronicity score is 12. The score may be used as a prognostic index. However, it is still doubtful for the value of the index and its reproducibility. Recently, a newer classification modified from the WHO classification has been proposed. (Table 2) This new classification claimed the reproducibility and correlation to the long-term

outcome (Table 3) (Weening, D'Agati, 2004)**(24)**. The incidence of class IV LN in Thai patients is higher than Caucasians (Parichatikanond, Francis, 1986).

**Table 2.** World Health Organization (WHO) morphologic classification of lupus nephritis (modified in 1982)

| | |
|---|---|
| Class I | Normal glomeruli |
| | a. Nil (by all techniques) |
| | b. Normal by light microscopy, but deposits by electron or immunofluorescence microscopy |
| Class II | Pure mesangial alterations (mesangiopathy) |
| | a. Mesangial widening and/or mild hypercellularity |
| | b. Moderate hypercellularity |
| Class III | Focal segmental glomerulonephritis (associated with mild or moderate mesangial alterations) |
| | a. With “active” necrotizing lesions |
| | b. With “active” and sclerosing lesions |
| | c. With sclerosing lesions |
| Class IV | Diffuse glomerulonephritis (severe mesangial, endocapillary or mesangiocapillary proliferation and/or extensive subendothelial deposits) |
| | a. Without segmental lesions |
| | b. With “active” necrotizing lesions |

| | |
|---|---|
| | c. With “active” and sclerosing lesions |
| | d. With sclerosing lesions |
| Class V | Diffuse membranous glomerulonephritis |
| | a. Pure membranous glomerulonephritis |
| | b. Associated with lesions of class II |
| | c. Associated with lesions of class III |
| | d. Associated with lesions of class IV |
| Class VI | Advanced sclerosing glomerulonephritis |

**Table 3.** International Society of Nephrology/Renal Pathology Society (ISN/RPS) 2003 classification of lupus nephritis.

| | |
|---|---|
| **Class I** | **Minimal mesangial lupus nephritis** |
| | Normal glomeruli by light microscopy, but mesangial immune deposits by immunofluorescence |
| **Class II** | **Mesangial proliferative lupus nephritis** |
| | Purely mesangial hypercellularity of any degree or mesangial matrix expansion by light microscopy, with mesangial immune deposits |
| | May be a few isolated subepithelial or subendothelial deposits visible by immunofluorescence or electron microscopy, but not by light microscopy |
| **Class III** | **Focal lupus nephritis[a]** |
| | Active or inactive focal, segmental or global endo- or extracapillary glomerulonephritis involving <50% of all glomeruli, typically with focal subendothelial immune deposits, with or without mesangial alterations |
| Class III (A) | Active lesions: focal proliferative lupus nephritis |
| Class III (A/C) | Active and chronic lesions: focal proliferative and sclerosing lupus nephritis |
| Class III (C) | Chronic inactive lesions with glomerular scars: focal sclerosing lupus nephritis |
| **Class IV** | **Diffuse lupus nephritis[b]** |
| | Active or inactive diffuse, segmental or global endo- or extracapillary glomerulonephritis involving ≥50% of all glomeruli, typically with diffuse subendothelial immune deposits, with or without mesangial alterations. This class is divided into diffuse segmental(IV-S) lupus nephritis when ≥50% of the involved glomeruli have segmental lesions, and diffuse global (IV-G) lupus nephritis when ≥50% of the involved glomeruli have global lesions. Segmental is defined as a glomerular lesion that involves less than half of the glomerular tuft. This class includes cases with diffuse wire loop deposits but with little or no glomerular proliferation |
| Class IV-S (A) | Active lesions: diffuse segmental proliferative lupus nephritis |
| Class IV-G (A) | Active lesions: diffuse global proliferative lupus nephritis |
| Class IV-S (A/C) | Active and chronic lesions: diffuse segmental proliferative and sclerosing lupus nephritis |
| | Active and chronic lesions: diffuse global proliferative and sclerosing lupus nephritis |
| Class IV-S (C) | Chronic inactive lesions with scars: diffuse segmental sclerosing lupus nephritis |
| Class IV-G (C) | Chronic inactive lesions with scars: diffuse global sclerosing lupus nephritis |
| **Class V** | **Membranous lupus nephritis** |
| | Global or segmental subepithelial immune deposits or their morphologic sequelae by light microscopy and by immunofluorescence or electron microscopy, with or without mesangial alterations |
| | Class V lupus nephritis may occur in combination with class III or IV in which case both will be diagnosed |
| | Class V lupus nephritis show advanced sclerosis |
| **Class VI** | **Advanced sclerosis lupus nephritis** |
| | ≥90% of glomeruli globally sclerosed without residual activity |

[a] Indicate the proportion of glomeruli with active and with sclerotic lesions.
[b] Indicate the proportion of glomeruli with fibrinoid necrosis and/or cellular crescents.
Indicate and grade (mild, moderate, severe) tubular atrophy, interstitial inflammation and fibrosis, severity of arteriosclerosis or other vascular lesions.

## 2. Overview of proteomics

The genome is the total set of genes (total DNA content) carried by an organism while the proteome is the total set of all the proteins expressed by a cell at any time. Since protein is the complement of an organism in genome. We define proteomics known as the study of gene expression at a functional level. Genes are the unit of genetics that control the progression of an individual and provide information for making the proteins that form each of us and our individual characteristics.

Although genes have usually been the primary area of focus at the molecular level, it is the proteins expressed that do most of the work in carrying out cellular processes and perform most life functions. For this reason, many researchers are turning their attention from genomics to proteomics to study the cellular and molecular basis of health, disease, and other life functions.

The term “proteomics” is a very modern concept defined as the large scale study and global analysis of all the proteins expressed by any cell or tissue under any conditions, with a focus on their structure and function are modified within the cell or tissue. Proteomics steps forward us from sequence of a protein to its biological role. It is the science that studies the proteins in general and also specific changes in their expression that result from various environmental conditions and/or disorders. The functional approach of proteomics helps to understand which set of protein is responsible for a given phenotype. While we expect cell that stay under different environmental condition and different organisms may be express different proteins, the cellular expression of enzymes for the same spices. The protein signature is specific group of proteins expressed by an organism under specific conditions. There are many different aspects of proteomics. One is the structural approach which

focuses on the molecular structure of proteins and relates this information to the database of identified genes.

**2.1 Mass spectrometry-based proteomic technologies**

Mass spectrometry (MS) is mostly used as a method for protein identification(Feng, Liu, 2008). There are two main components of a mass spectrometry(Mischak, Julian, 2007); 1) the ionization technique and 2) the mass analyzer. Matrix-assisted laser desorption/ionization (MALDI) and electrospray ionization (ESI) are widely used ionization technique to volatilize and ionize protein and peptides for mass spectrometry. There are four types of mass analyzers; 1) time-of-flight (TOF), 2) ion-trap (IT), 3) quadrupole(Q) and 4) fourier transform ion cyclotron resonance (FTICR). In general, four different types of proteomic technologies are used in proteomics; 1) two-dimensional gel electrophoresis coupled to mass spectrometry, 2) surface-enhanced laser desortion/ionization coupled to mass spectrometry, 3) liquid chromatography coupled to mass spectrometry, 4) capillary electrophoresis coupled to mass spectrometry. Their advantage and disadvantage are summarized in Table 4.

2.1.1 Two-dimensional gel electrophoresis

Two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) is common technique used in protein study. It has been the technique of choice for analyzing the protein of cells, tissues and fluids as well as for studying the changes in global patterns of gene expression. This technique work couple with mass spectrometry which can then be used to identify the proteins. The basic of 2DE proteomics methodology includes this step (figure 2): solubilization of protein from sample, separation of the protein by 2DE, analyzation of 2D gels and computer-assisted

analysis of spot patterns, determination of specific attributes of the proteins of interest by MS and, searching of databases with these attributes to identify the proteins (Cho, Lee, 2003).

In 2D-PAGE, proteins are separated by two properties: isoelectric point (pI) and molecular weight (MW). The isoelectric point is the pH at which the protein has no net charge. In the first dimension, which is known as isoelectric focusing (IEF), proteins are putted to a strip containing an immobilized pH gradient (called IPG strips) and the proteins migrate to their isoelectric point when apply an electric field. As proteins migrate to their respective isoelectric points, they pick up or lose protons. As they continue to migrate, the net charge on the proteins and their mobility decreases and eventually they come to a point where their net charge is zero and they stop moving. Proteins was separated by IEF are further separated following by SDS-PAGE (Sodium dodecyl sulfate-polyacrylamide gel electrophoresis). The transition from first to second dimension involves 2 steps. The first step involves the equilibration of IPG strips in a buffer containing SDS, a detergent that imparts negative charge to the protein that is proportional to the mass of a protein. In addition, the proteins that have been separated by IEF are also reduced and alkylated using DTT (dithiothreitol) and iodoacetamide (IAA). DTT cuts the disulfide bonds (R-S-S-R) found in proteins. The resulting –SH groups are then alkylated with iodoacetamide, which attaches an alkyl group to the –SH (R-S-CH2-CONH2) to prevent reorganization of the disulfide bonds as shown in figure below (Figure 3).

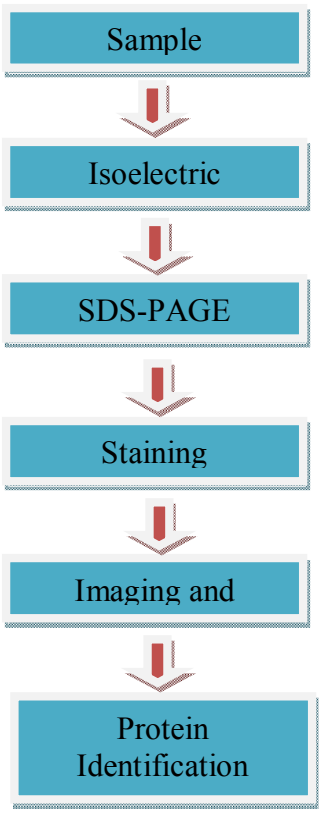


**Figure 2**. Flow chart describes the proteomic process of moving from a cellular homogenate to identification of a proteomic signature

The treated IPG strips are then fixed in the top of an SDS-PAGE gel. An electric current is then applied to the system, causing the proteins to migrate from the IPG strip into the SDS-PAGE gel, where they are separated by size. Proteins move through the porous gel with small molecules moving more rapidly than the larger ones. Polyacrylamide is a cross-linked polymer of acrylamide (Figure 4). Acrylamide is the material used for

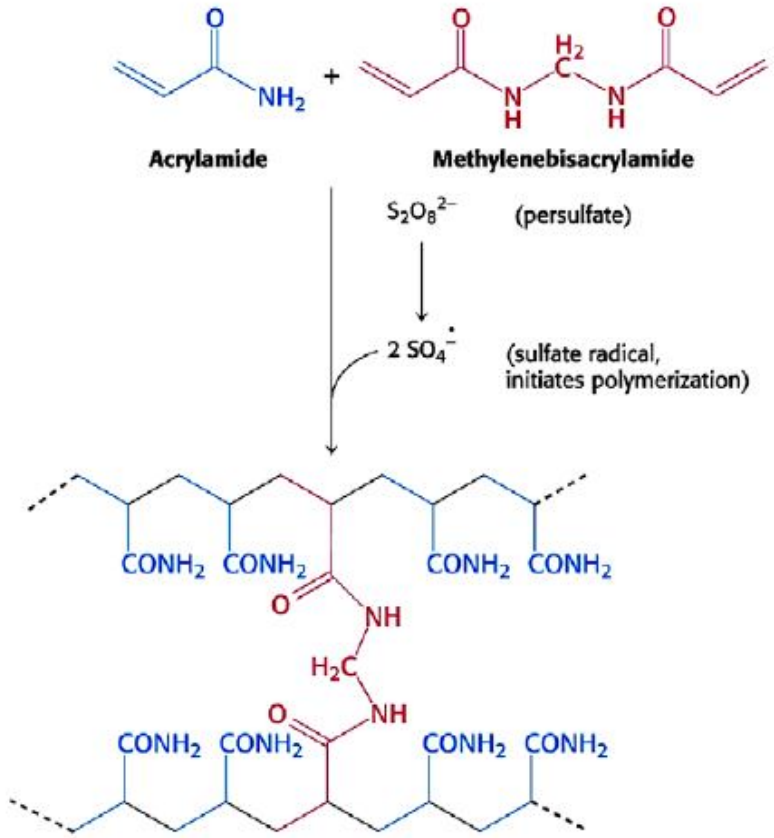


**Figure 3.** Reduction and Alkylation step in the process 2-DE

preparing gels for electrophoresis and separates proteins by their molecular size. Ammonium persulfate is the polymerizing agent which when added to acrylamide gel mixed with bisacrylamide, forms a network of cross-linked polymer. The pore size in the gel is inversely related to the amount of acrylamide used. Gels containing a high percentage of acrylamide are used to resolve small proteins, whereas low percentage acrylamide gels are used to resolve large protein. Typical polyacrylamide concentrations range from 5% to 25%. Gels can also be prepared with a gradient in polyacrylamide concentration (e.g. 8-16%), which broadens the MW range for protein separation on a single gel.

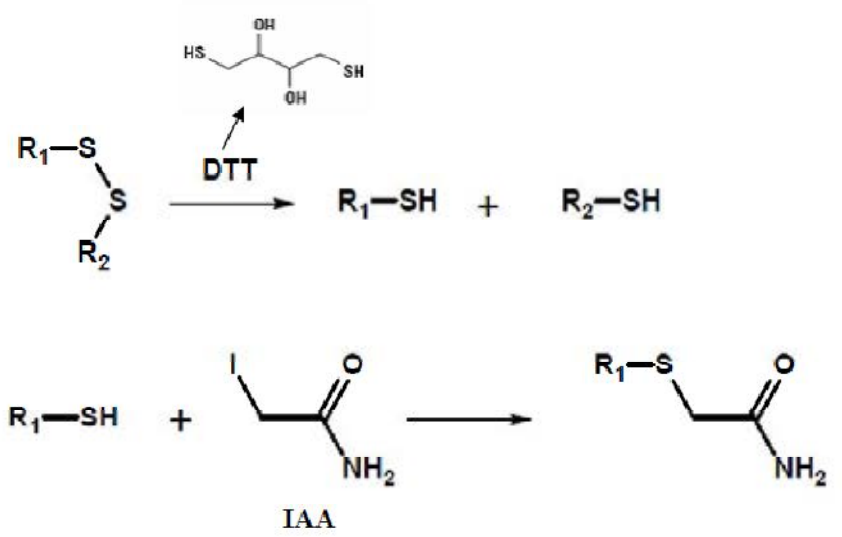


**Figure 4**. Polyacrylamide, a cross-linked polymer of acrylamide

2.1.2 Surface-enhanced laser desortion/ionization coupled to mass-spectrometry (SELDI-TOF)

SELDI decreases the complexity of a biological sample by difference interactions of peptide with different active surface such as hydrophilic, reversed-phase, lectin or antibody affinity. After binding, only a small fraction of peptide can bind to the surface of the SELDI chip, depending on the concentration, pH, salt content, presence of interfering compounds like lipids while the unbound samples are washed away (Huang, Clifton, 2009). A sample surface is mixed with matrix to absorb energy and to allow vaporization and ionization by laser for further MS detection. SELDI is used to detect biomarkers in a variety of disease because of its ease of operation and its high throughput (Najam-ul-Haq, Rainer, 2007). On the other hand, low reproducibility and comparability of dataset are limit in this technique.

2.1.3 Liquid chromatography coupled to mass spectrometry (LC/MS)

LC provides a great fractionation method that is compatible with any type of mass spectrometers. This method separates a lot of analytes on an LC-column with high sensitivity. The column contains the materials with various physical, chemical, and immunological properties. When the sample dissolves in a solvent and then moves pass the column, the peptides in the sample can be separated by elution at different time points depending on their separation characteristics. A separation as sequence using different matrices in two independent steps provides a multidimensional fractionation that can generate large amount of information. Two dimensional liquid-phase fractionation approaches are well-suited for in depth analysis of body fluid such as urine. However, limitation is time-consuming and sensitive to interfering compounds and precipitation of analytes on LC-column. Thus, application of LC/MS is not suitable to clinical analysis (Liu, Chen, 2009, Wu, Hu, 2008).

2.1.4 Capillary electrophoresis coupled to mass spectrometry

CE/MS uses MS-based approach for the proteomics analysis of body fluids. This technique separates proteins with high resolution based on their pass though a buffer-filled capillary column in an electrical field. The advantage of CE/MS is listed: 1) it provides fast and robust separation using an inexpensive capillaries, 2) it is well-matched with many buffer and analytes, 3) it provides a stable constant flow that may avoid interfering following MS detection by gradients. The disadvantage of CE/MS is that it is not suitable for the analysis of molecular weight more than 20 kDa as they tend to precipitate in the acidic buffers that are used for CE/MS analysis and only

small sample volume can be loaded onto the capillary leading to a lower selectivity compared with LC(Mischak, Julian, 2007).

**2.2 Urinary proteomics in lupus nephritis.**

The biomarkers were strongly correlated with renal disease activity. In adult lupus patients, Mosley et al., identified two mass of protein by using SLEDI-TOF MS which 2 mass of protein had a sensitivity and specificity for active lupus nephritis of 92% each (Mosley, Tam, 2006). In a study including only children and young adult with SLE or juvenile idiopathic arthritis (JIA), 8 peaks mass candidate of protein was found. There are 2.7, 22, 23, 44, 56, 79, 100 and 133 kDa (Suzuki, Ross, 2007). Subsequently, Suzuki and co-worker identified the 23 kDa band as lipocalin type prostaglandin D synthetase (L-PGDS), the 56 kDa band as alpha-1-acid-glycoprotien (AGP), the 79 kDa as transferring and the 133 kDa band as ceruloplasmin (Suzuki, Wiers, 2009). Zhang et al., identidied 27 differentially expressed protein ions in different phases of a lupus nephritis flare. Three potential low-molecular weight biomarkers namely the 20 and 25 amino acid isoforms of hepcidin, fragment of alpha-1-antitrypsin and albumin. The hepcidin protein appears to have a significant relationship with proinflammatory cytokine in LN (Zhang, Jin, 2008)

**Table 4**. Summary of Advantages and Limitations of Different Proteomic

| Method | advantages | limitations |
|---|---|---|
| 2-DE | -Good resolution | -Time-consuming<br>-Not for hydrophobic<br>-Not easy to detected low molecular weight |
| SEIDI-TOF | -Easy to use<br>-High throughput<br>-Low sample volume required | -Not easy to reproducibility<br>- Low resolution and sensitivity<br>-Restricted of selected peptide<br>-Interpretation of data is easy but subsequent use is difficult |
| LC/MS | -Automation<br>-Multidimensional<br>-High-throughput capacity | -Sensitive toward interfering<br>-High capacity costs<br>-Requires sequence databases for analysis compound |
| CE-MS | - High resolution, high selectivity<br>- Fast separation, low cost | - Not suited for the analysis of<br>-high molecular weight Protein |

## CHAPTER III

# Methods

### 1. Study design

16 patients with SLE (5 active, 5 inactive, 3 responder and 3 nonresponder) that meet the classification criteria according to the American College of Rheumatology were included in this study (Tan, Cohen, 1982). All patients were biopsy-proven class III or IV LN according to the 2003 International Society of Nephrology/Renal Pathology Society Classification (Weening, D'Agati, 2004). All patients were required to have any combination of clinical criteria present: 1) urine protein creatinine index > 0.5 g/day for active LN and < 0.5 g/day for inactive LN, 2) active-urine sediments (red blood cells> 5, or white blood cells > 5 per high-power field) and inactive-urine sediments (red blood cells < 5, or white blood cells < 5 per high-power field). For response and non-response group, patients were treated with Mycophenolate mofetil (MMF) 1.0-2.0 g/day for 6 months based on the physician's prescription. This criteria was defined for response to treatment: 1) have a normal or increased estimated glomerular filtration rate by 25% if the baseline estimated glomerular filtration rate was abnormal, (2) have a 50% reduction of 24-h urine protein resulting in a level > 2 g/day (2006). We excluded patients who were pregnant or had serum creatinine more than 3 mg/dL.

All experiments involving human contact was performed with patient written consent and approved by the Ethics Committee for Human Research, Faculty of Medicine, Chulalongkorn University, Bangkok, Thailand.

### 2. Urine collection and precipitation

Urine samples were collected LN patients (inactive, active, responder and

nonresponder). The urine sample was centrifuged at 1000g, 4 º C for 5 min to clear debris. The supernatant was precipitated by 75% ethanol and mixed (Thongboonkerd, Chutipongtanate, 2006). Samples were incubated on ice for 10 min following by centrifugation at 12,000g for five minute. The pellet was resuspended in a sample buffer containing 7 M urea, 2 M thiourea, 4% CHAPS (3[(3-cholamidopropyl) dimethylammonio]-1-propanesulfonate), and 30 mM Tris-base. The solution was dialyzed against 18 Megaohm((cm (dI) water (at 4°C overnight) and then concentrated by using amicon-15. Protein concentration of each sample was measured by spectrophotometry using a Biorad protein microassay.

**3. Two dimensional polyacylamide electrophoresis**

Immobilized pH gradient (IPG) strips, linear pH 3-10,7 and 13 cm long (Amersham Biosciences) were rehydrated overnight with 150 and 200 respectively, (µg proteins using rehydration buffer containing 8 M urea, 2% CHAPS, 2% ampholytes and bromophenol blue. The first dimensional separation (IEF) was performed in Ettan IPGphor II Isoelectric Focusing Unit (Amersham Biosciences) at 20ºC. After completion of the IEF, the samples were equilibrated with a buffer containing 130 mM DTT, 50 % glycerol, 50 mM Tris base, 2% SDS, 0.002% bromophenol blue and then with another buffer containing 135 mM iodoacetamide, 50% glycerol, 50 mM Tris base, 2% SDS, 0.002% bromophenol blue. The focused IPG strips were then transferred onto 12.5% acrylamide slab gel (8x8 cm) for strip 7 cm and (16 x 16 cm) for strip 13 cm and the second dimensional separation was performed in SE 600 Ruby system (Amersham Biosciences) with the current of 30 (µA/gel for 3.5 h.The gel slabs was fixed in 10% acetic acid and 40% ethanol for 30 minutes and stained with Coomassie Brilliant Blue R-250 stain one day and washed

with DI water overnight.

**4. Matching and Quantitive volume analysis.**

Image Master 2D Platinum software was used for detecting, matching and analysis of Image Master 2D Platinum (GE Healthcare) software was used for matching and analysis of protein spots in 2-D gels. Parameters used for spot detection were i) minimal area 5 pixels ii) smooth factor 2, and iii) saliency 10. A reference gel was created from an artificial gel combining all of the spots presenting in different gels into one image. Spots were quantitated as a fraction of the total volume of protein spots on the gel. One typical control gel with the most protein spots was set as reference gel, to which all the other gels will be matched. Match rates of each group of gels was calculated automatically by the software. Class report analysis was run on the software and each spot group with a between-class ratio of more than two or less than 0.05 was statistically analyzed using the unpaired t-test, two-tailed.

**5. In gel tryptic digestion.**

Differentially expressed protein spots were excised from 2-DE, washed twice with 200 µl 50% acetonitrile (ACN)/25 mM $NH_4HCO_3$ buffer (pH 8.0) and then incubated at room temperature for 10 minutes. After that the solution was discarded and repeated this process twice. The gels were dried by using speedvac. 100 µl of 10 mM DTT in 25 mM $NH_4HCO_3$ dried gels following this reaction to proceed at 56º C for 1 hour. After reduction, 100 µl of 55 mM idoacetamind to the gel pieces and allowed the reaction to proceed in the dark for 45 minutes. The gel pieces were washed with 200 µl 50% acetonitrile (ACN)/ 25 mM $NH_4HCO_3$ and speedvec and digested with 45 ul of sequence modified trypsin (Progema, Madison, WI) at 37 ◦C overnight (Shevchenko, Wilm, 1996). Peptides were extracted with 25 mM $NH_4HCO_3$

in 50% ACN. The extracts were evaporated in a vacuum centrifuge and the pellets resuspended in 0.1% TFA. The peptides were analysed on an UltiMate™ NanoLC system (Dionex-LC Packings) coupled to a Bruker micrOTOF-Q II™, equipped with an online nanoESI source. The complete LC-MS setup was controlled by HyStar™software. The instrument was connected on-line with a PepMap C18 Nanocolumn (75 μm×150 mm (LC Packings). A linear 60 min gradient was achieved from 100% solvent A (95:5 H2O/ACN 0.1% FA) to 60% solvent B (20:80 H2O/ACN 0.1% FA) at a flow rate of 300 nl/min. As peptides eluted from the nanocapillary column, they were electrosprayed into the nanoESI source. Spray voltage and capillary temperature were set at 1500-2500 V and 160 °C respectively. After that Bruker proprietary algorithm was used to detect the charge state of percursor ion during acquisition. Ion parent and fragmentation mass spectra were analysed using DataAnalysis software (Bruker Daltonics).

The obtained amino acid sequences were used to search through SWISSPROT database by the Mascot search engine (http://www.matrixscience.com). The search was limited with mass tolerances of 100-150 ppm. Only one missed cleavage per peptide was allowed and cysteine residues were assumed to be carbamidomethylated with acrylamide adducts and methionine residues were in oxidized form. Significant matching required protein scores > 35 ($p<0.05$)

**6. Validation of the identified proteins**

Spot urine samples from 56 Lupus nephritis (active LN = 30, inactive=26), 8 healthy controls and 6 nephrotic diseases (biopsy confirmed diagnosis of Focal Segmental Glomerulosclerosis) were collected for validating by using Enzyme-linked immunosorbent assay (ELISA). Urinary excretion of ZA2G and PGDS were

measured using a commercial ELISA kit from Biovendor, Brno, zech Republic and Cayman Chemical according to the manufacturer's instructions. Spot urine samples from responder LN (n=6) and nonresponder LN (n=5) were collected and validated by ELISA. Urinary excretion of A1M and CD 59 glycoprotein were measured using a commercial ELISA kit from Abcam, Cambridge, USA and Mybiosource, San diego, California according to the manufacturer's instructions.

**7. Statistical analysis**

The values were expressed as mean ± SEM. The differences were assessed by one-way ANOVA and parametric Tukey post doc test, Student's t-test, and multivariate analyses using the SPSS software version 11.5 (SPSS Inc., Chicogo, IL, USA).

# CHAPTER IV

# Results

## Proteome Profiling and Protein identification

### 1.1.1 Active and Inactive Patient's characteristics

In this study, we had two groups of patients for urinary proteomics; 1) Active and inactive LN group. For 2-DE, ten patients were active (n=5) versus inactive (n=5) LN. Four active LN patients had class IV and one patient had class III+IV. Three patients were class III. Moreover, we had sample for validating in an independent set. Among fifty-nine patients, there were 30 active, 26 inactive, 6 other glomerular diseases and 8 healthy controls. Among groups, there were no difference in age, serum creatinine and estimated glomerular rate filtration (eGFR). The active LN had higher scores of SLEDAI-2K, renal SLEDAI (rSLEDAI), and higher levels of urine protein creatinine index (UPCI). Table 5 showed summary of all patients' characteristics. Twenty-five active LN patients had ISN/RPS class III or IV lupus nephritis. Three active patients and one patient had class V+IV and class III+IV respectively. All active patients were treated with oral Prednisolone.

### 1.1.2 Proteome Map of urine in LN patients

Urine samples from five active LN, five inactive LN, three responder and 3 nonresponder were collected and precipitated with 75% ethanol. Two hundred microgram protein was resolved and visualzed with coomassie blue in 2-DE as shown in figure 5. Image master 2D platinum software was used for matching and analysis of protein spots on gels. We selected a gel with good protein resolution, excellent

protein stain, and minimal image distortion to be the reference gel. For ten of 2D gels were generated from the urine of five active and five inactive respectively as figure 5A and 5B shown. There were about 300-400 protein spots being detected and the relative volume of each spot was acquired through the software. Thirty-eight protein spots in active LN gel as reference for matching were excised and subjected to ESI/MS/MS (figure 6). Locations of all these identified proteins on 2-D gels were showed their accession number from SWISSPROT, percent of sequence coverage, theoretical isoelectric points (pI), and molecular weights (Mw) as summarized in Table 6.

**1.1.3 Differentially expressed spots**

We compared the relative volumes between active and inactive LN and found that 14 were spots differentially expressed and 2 spots were present in active LN samples but absent in inactive samples as shown in Table 7. Figure 7 showed the relative volume spot had significantly differential expressed between groups.

We classified all differentially expressed protein into different functional group on the basis of their major cellular functions, including 1) defense/immunity protein such as Beta-2-microglobulin, Ig Kappa chain C, Ig kappa chain V-III HAH region and Ig kappa chain V-III SLE region K, 2) enzyme modulator; alpha-1-antitrypsin, 3) extracellular matrix protein; Alpha-2-HS glycoprotein, 4) isomerase; Prostaglandin-H2-D isomerase, 5) protease; haptoglobin, 6) receptor; Zinc alpha-2-glycoprotein 7) transfer/carrier protein; retinol-binding protein 4, albumin and serotransferrin and 8) transporter; tranthyretin as shown in figure 8

### 1.1.4 Validation of protein expression

**Urinary ZA2G and PGDS were increased in active LN patients.**

We selected ZA2G and PGDS for further validation because ZA2G has the highest ratio between active and inactive LN (6 folds difference) and PGDS was present only in active LN. Moreover, PGDS plays a role in inflammatory response and chemoattractant properties. We validated two proteins in an independent set of samples by using enzyme-linked immunosorbent assay (ELISA). Urinary excretion of ZA2G were significantly up-regulated in active LN as compared with inactive LN ($p < 0.0001$) and healthy control ($p < 0.0001$) but not significantly different from other glomerular diseases (Figure9). Urinary excretion of PGDS in active LN was significantly higher than others (inactive LN patients ($p<0.0004$), healthy control ($p<0.002$) and other glomerular diseases ($p<0.0001$).

After treatment at 6 month, we classified patient into 2 groups: 1) responder and 2) nonresponder. The change in urinary excretion of PGDS in pretreatment patient and after treatment patient during 3 and 6 month as shown in figure 10. We found a reduction of the urinary excretion of PGDS at 6 month when compare with 0 month in responder LN whereas to be increased at 6 month in nonresponder. However, urinary excretion of PGDS did not have significant change between 0 and 6 month.

We next evaluated a correlation between urinary excretion both of PGDS, ZA2G with UPCI, SCr, eGFR, SLEDAI-2K and rSLEDAI. We analyzed the data using the Pearson's bivariate correlation method as shown in Table 7. Urinary excretion of PGDS and ZA2G were significantly correlated with proteinuria ($R^2$=0.45; $P < 0.01$ and $R^2$= 0.65;$P< 0.05$ respectively) shown as figure 11A and 11B.

Moreover, ZA2G showed a significant inverse correlation with eGFR ($R^2$=-0.52; P< 0.01) (figure 11C).

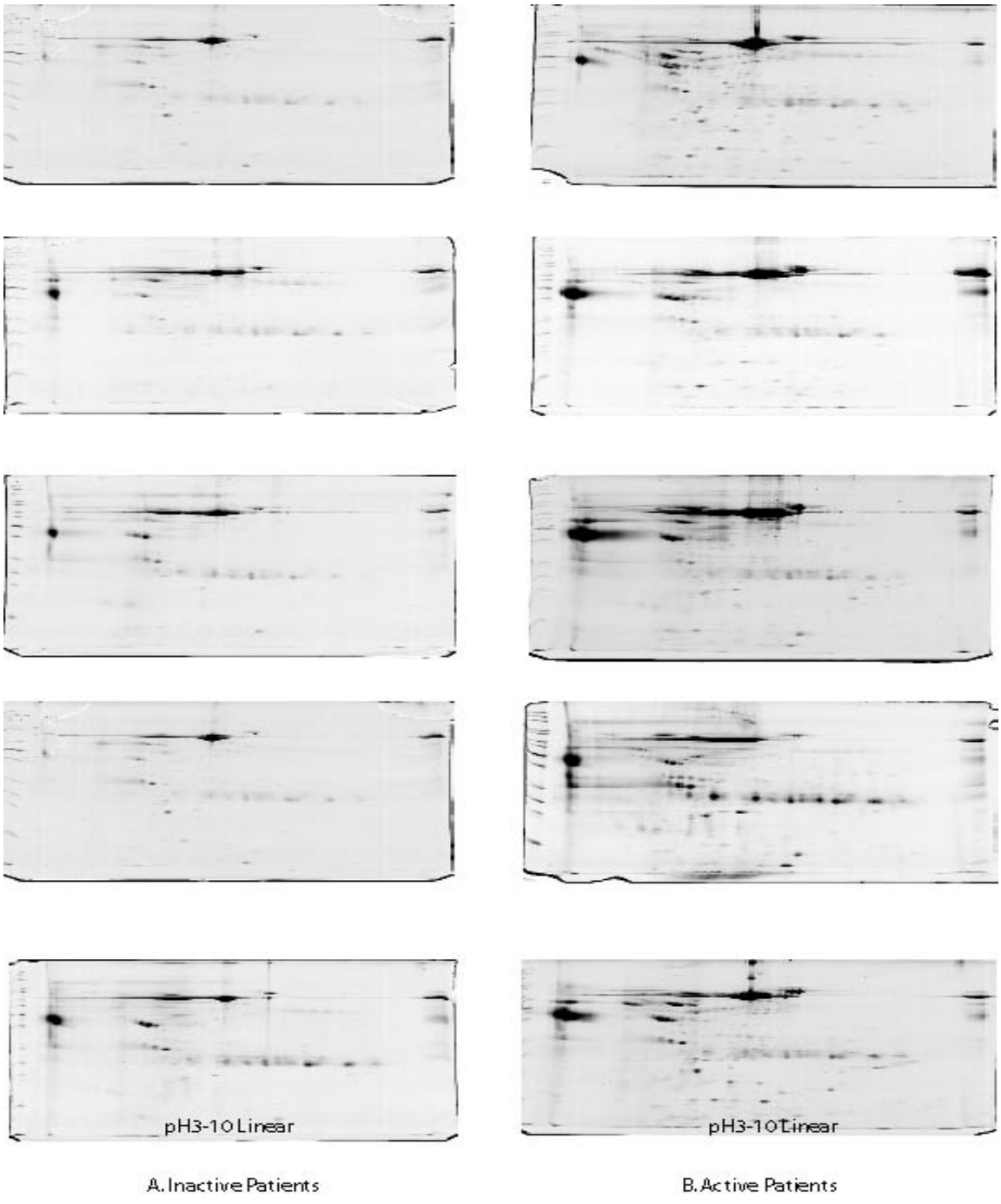


**Figure 5**. Two-DE gels from active and inactive LN. Proteins were resolved using a linear pH 3–10 gradient in the first dimension and by SDS-PAGE on a 12.5% acrylamide gel in the second. Gels were stained with coomassie blue. Patients were defined as active LN (A) and inactive LN (B), respectively.

**Table 5.** Demographics of active and inactive LN patients.

| Patient characteristic | Active LN | Inactive LN | Healthy controls | Nephrotic diseases |
|---|---|---|---|---|
| Sex, female:male | 28:2 | 26:0 | 8:0 | 5:1 |
| Age (year ) | 32.77±1.42 | 34.52±1.46 | 26.66±1.66 | 35.33±4.44 |
| Serum Creatinine(mg/dl) | 0.92±0.05 | 0.80±0.06 | 0.64±0.05 | 1.02±2.80 |
| eGFR(MDRD) (ml/min) | 78.27±4.82 | 98.84±6.67 | 113.33±11.14 | 86.75±13.13 |
| Urine protein creatinine index | 3.37±0.42 | 0.79±0.06 | 0.09±0.01 | 5.78±1.40 |
| White blood cell (cell/hyperfield) | 25.50±2.64 | 2.31±0.59 | 0 | 1.20±0.20 |
| Red blood cell (cell/hyperfield) | 24.55±4.47 | 6.92±3.97 | 0 | 14.70±9.02 |
| SLEDAI-2K | 15.36±1.88 | 2.17±0.76 | N/A | - |
| Renal SLEDAI | 11.60±0.62 | 2.24±0.57 | N/A | - |
| Steroid dosage(mg/day) | 11.86±1.92 | 2.30±0.51 | N/A | N/A |

**Table 6**. The identified proteins from active LN urine as reference

| No | Protein | Accession number | pI | Molecular weight | Score | Seq Cov% |
|---|---|---|---|---|---|---|
| 1 | Serotransferin | P02787 | 6.81 | 79.29 | 1877 | 66 |
| 2 | Albumin | P02768 | 5.92 | 71.32 | 642 | 65 |
| 3 | Albumin | P02768 | 5.92 | 71.32 | 404 | 55 |
| 4 | Alpha-B1-glycoprotein | P02763 | 5.56 | 54.79 | 980 | 57 |
| 5 | Alpha-2-HS glycoprotein | P02765 | 5.43 | 40.09 | 592 | 65 |
| 6 | Kininogen-1 | P01042 | 6.34 | 72.99 | 505 | 30 |
| 7 | Alpha-1-antitrypsin | P01009 | 5.37 | 46.78 | 1548 | 69 |
| 8 | Haptoglobin | P00738 | 6.13 | 45.81 | 347 | 30 |
| 9 | Albumin | P02768 | 5.96 | 71.32 | 577 | 46 |
| 10 | Zn-alpha2-glycoprotein | P25311 | 5.71 | 34.47 | 142 | 37 |
| 11 | Zn-alpha2-glycoprotein | P25311 | 5.71 | 34.47 | 430 | 52 |
| 12 | Zn-alpha2-glycoprotein | P25311 | 5.71 | 34.47 | 1028 | 55 |
| 13 | Zn-alpha2-glycoprotein | P25311 | 5.71 | 34.47 | 941 | 61 |
| 14 | Haptoglobin-related protein | P00739 | 6.63 | 39.51 | 70 | 21 |
| 15 | Haptoglobin-related protein | P00739 | 6.63 | 39.51 | 155 | 38 |
| 16 | Inter-alpha-trypsin inhibitor heavy chain H4 | Q14624 | 6.51 | 103.52 | 592 | 14 |
| 17 | Alpha-1-microglobulin | P02760 | 5.95 | 39.89 | 479 | 32 |
| 18 | Alpha-1-microglobulin | P02760 | 5.95 | 39.89 | 545 | 32 |
| 19 | Alpha-1-microglobulin | P02760 | 5.95 | 39.89 | 600 | 32 |
| 20 | Ig Kappa chain C region | P01834 | 5.58 | 11.77 | 70 | 50 |

| | | | | | | |
|---|---|---|---|---|---|---|
| 21 | Ig Kappa chain C region | P01834 | 5.58 | 11.77 | 86 | 61 |
| 22 | Ig Kappa chain C region | P01834 | 5.58 | 11.77 | 284 | 80 |
| 23 | Ig Kappa chain C region | P01834 | 5.58 | 11.77 | 487 | 82 |
| 24 | Ig kappa chain V-III SLE region | P18135 | 7.74 | 14.18 | 175 | 36 |
| 25 | Ig kappa chain V-III HAH region | P01620 | 8.70 | 11.88 | 217 | 31 |
| 26 | Ig kappa chain V-III HAH region | P01620 | 8.70 | 11.88 | 215 | 39 |
| 27 | Ig Kappa chain C region | P01834 | 5.58 | 11.77 | 581 | 82 |
| 28 | Ig Kappa chain C region | P01834 | 5.58 | 11.77 | 390 | 82 |
| 29 | Ig kappa chain C region | P01834 | 5.58 | 11.77 | 108 | 61 |
| 30 | Membran-specific heparin sulfate Proteoglycan core protein | P98160 | 6.06 | - | 956 | 4 |
| 31 | Retinol-binding protein 4 | P02753 | 5.76 | 23.38 | 620 | 70 |
| 32 | Albumin | P02768 | 5.92 | 71.32 | 572 | 18 |
| 33 | Albumin | P02768 | 5.92 | 71.32 | 353 | 13 |
| 34 | Albumin | P02768 | 5.92 | 71.32 | 266 | 13 |
| 35 | Beta-2-microglobulin | P61769 | 6.06 | 13.82 | 38 | 26 |
| 36 | Unidentified | | | | | |
| 37 | Transthyrein | P02766 | 5.52 | 15.99 | 332 | 69 |
| 38 | Prostaglandin-H2-D isomerase (brain) | P41222 | 7.66 | 21.24 | 156 | 32 |

Seq Cov%; %sequence coverage [(number of the matched residues/total number of residues in the entire sequence)x100], pI; Isoelectric point

**Table 7**. Differently expressed protein between group

| spot No | Protein ID | Accession Number | pI/MW (KDa) | Protein score | % Seq Cov | % Vol relative(arbitrary unit) Active LN (Mean±SEM) | Inactive LN (Mean±SEM) | ratio A/I | p |
|---|---|---|---|---|---|---|---|---|---|
| **Plasma proteins** | | | | | | | | | |
| 1 | Serotransferrin | P02787 | 6.81/79.29 | 1877 | 66 | 5.69±0.57 | 1.99±0.73 | 3.01 | 0.004 |
| 4 | Alpha-B1-glycoprotein | P02763 | 5.56/54.79 | 980 | 57 | 18.33±1.92 | 11.10±1.39 | 1.65 | 0.018 |
| 8 | Haptoglobin | P00738 | 6.13/45.81 | 347 | 30 | 0.32±2.13 | 0.01±0.008 | 5.00 | 0.05 |
| 9 | Albumin | P02768 | 5.96/71.32 | 577 | 46 | 0.02±0.005 | 0.009±0.005 | 2.22 | 0.05 |
| 11 | Zn-alpha2-glycoprotein | P25311 | 5.47/71.34 | 430 | 52 | 0.76±0.07 | 0.23±0.03 | 3.30 | 0.001 |
| 12 | Zn-alpha2-glycoprotein | P25311 | 5.47/71.34 | 1082 | 55 | 0.79±0.06 | 0.04±0.01 | 2.35 | 0.0001 |
| 13 | Zn-alpha2-glycoprotein | P25311 | 5.47/71.34 | 941 | 61 | 0.18±0.08 | 0.03±0.01 | 19.75 | 0.05 |
| 24 | Ig kappa chain V-III SLE region | P18135 | 7.74/14.18 | 175 | 36 | 1.39±0.45 | 0.32±0.07 | 4.34 | 0.05 |
| 26 | Ig kappa chain V-III HAH region | P01620 | 8.70/11.88 | 217 | 31 | 0.76±0.16 | 0.16±0.06 | 4.75 | 0.02 |
| 29 | Ig kappa chain C region | P01834 | 5.58/11.77 | 108 | 61 | 0.02±0.009 | 0.009±0.005 | 2.22 | 0.05 |
| 35 | Beta-2-microglobulin | P61769 | 6.06/13.82 | 38 | 26 | 0.03±0.007 | 0.006±0.003 | 5.00 | 0.05 |
| **Matrix protein** | | | | | | | | | |
| 5 | Alpha-2-HS glycoprotein | P02765 | 5.43/40.09 | 592 | 65 | 0.80±0.01 | - | DIV/0 | - |

| | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|
| **Protease inhibitor** | | | | | | | | | |
| 7 | Alpha-1-antitrypsin | P01009 | 5.37/46.78 | 1548 | 69 | 2.52±0.80 | 0.89±0.81 | 2.83 | 0.013 |
| **Proteolytic lipocalin** | | | | | | | | | |
| 31 | Retinol-binding protein 4 | P02753 | 5.76/23.38 | 620 | 70 | 0.37±0.15 | 0.05±0.02 | 7.4 | 0.01 |
| **Thyroid hormone binding** | | | | | | | | | |
| 37 | Transthyretin | P02766 | 5.52/15.99 | 332 | 69 | 0.10±0.03 | 0.06±0.01 | 1.67 | 0.05 |
| **Enzyme protein** | | | | | | | | | |
| 38 | Prostaglndin-H2-D isomerase (brain) | P41222 | 7.66/21.24 | 156 | 32 | 0.02±0.006 | 0.00±0.00 | DIV/0 | - |

% volume relative; [(volume of spot/volume over all the spots in the gel)x100], pI/MW; isoelectric point/molecular weight, A/I; active LN/Inactive LN, KDa; kilodalton, DIV/0; divied by zero

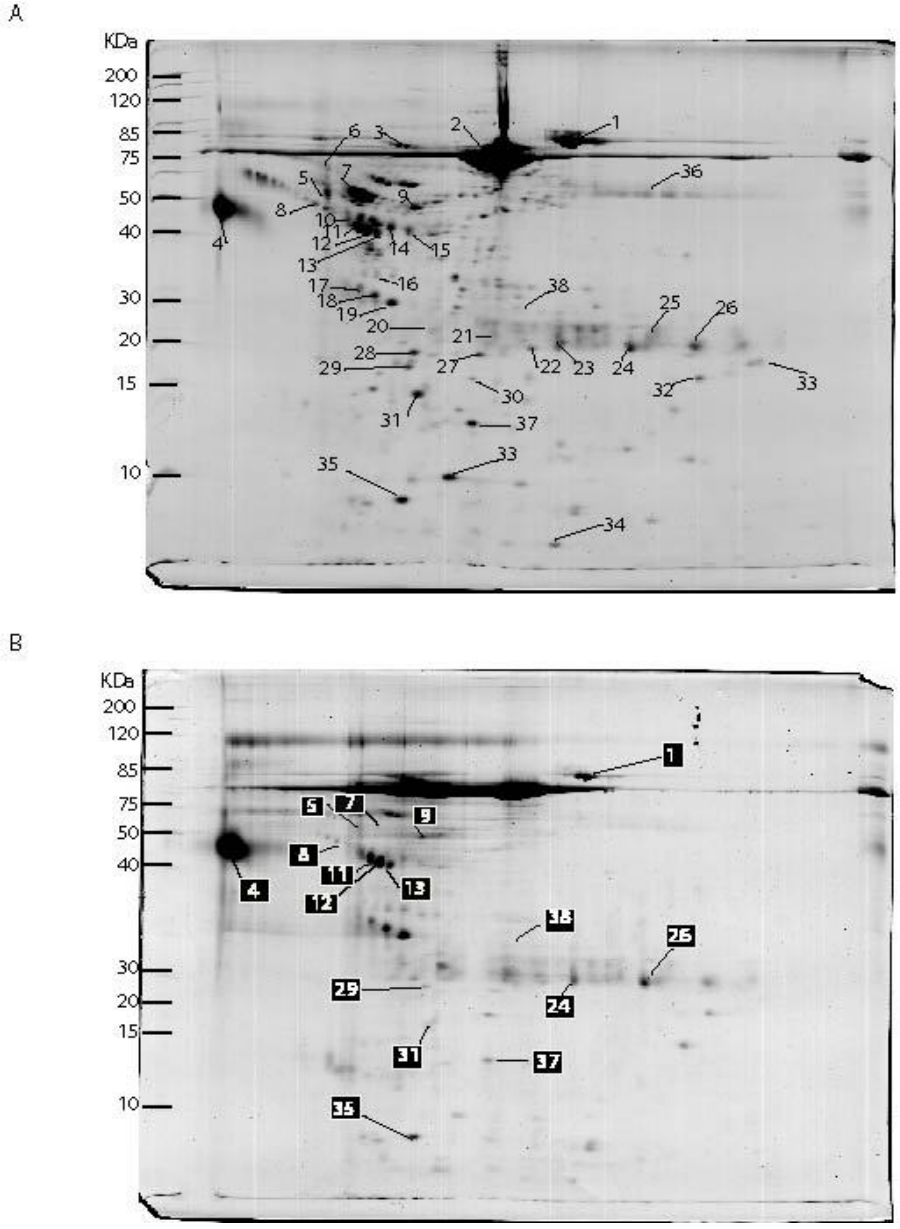


**Figure 6**. The reference 2-DE gels from active (A) and inactive LN (B). Protein were resolved using a linear pH 3-10 gradient in the first dimension and by SDS-PAGE on a 12.5% acrylamide gel in the second. Gels were stain with coomassie blue. Protein spots were identified by LC/MS/MS. (A) total 38 spots were identified in the active LN (summarized in table2). Boxes in (B): Sixteen differentially expression spots using Image Master 2D Platinum software (Amersham Bioscience) and student unpaired T-test.

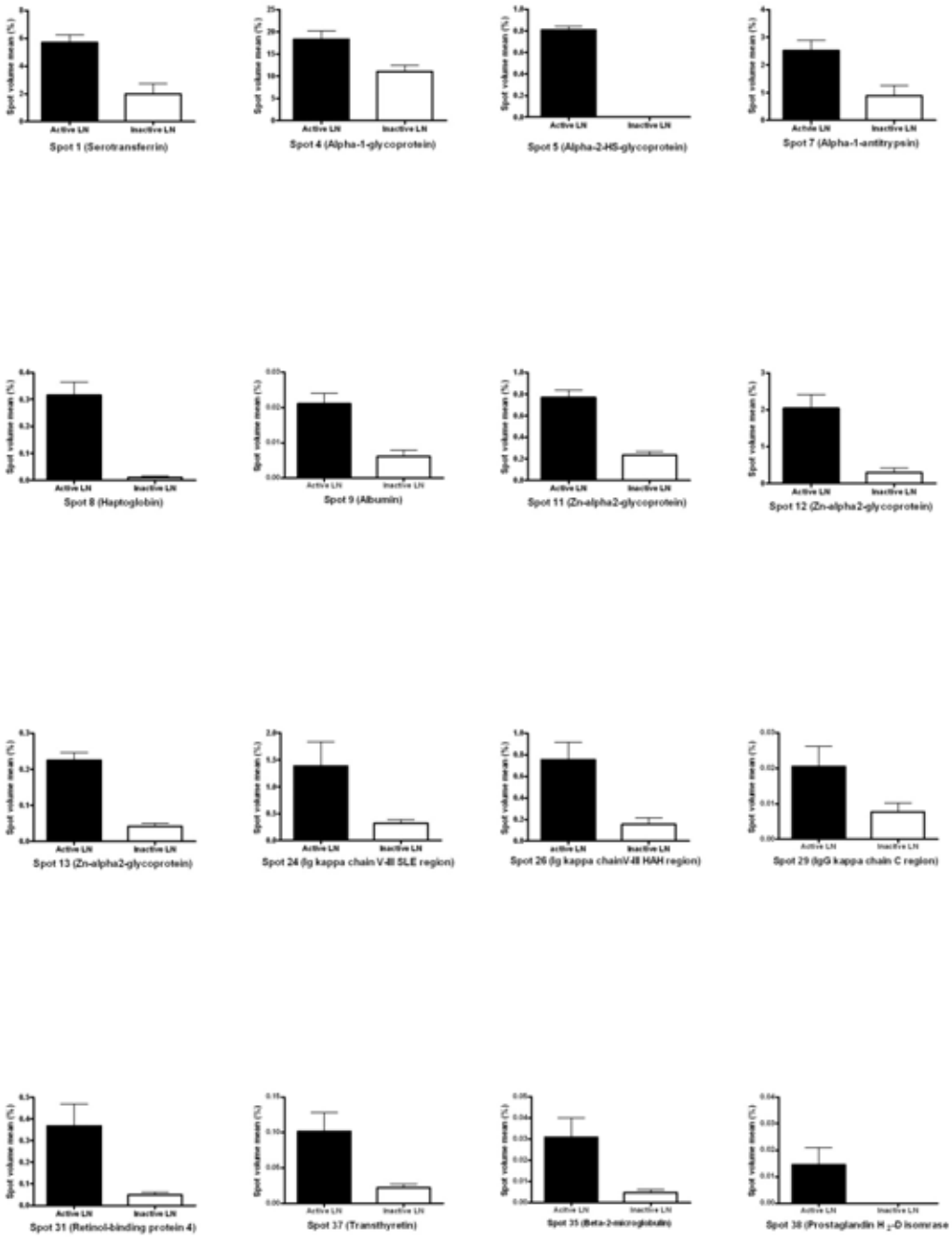


**Figure 7**. Histogram represents of the relative volume mean (%) of 16 spots that had significantly differential expression. Value of plotted are mean +/- SEM.

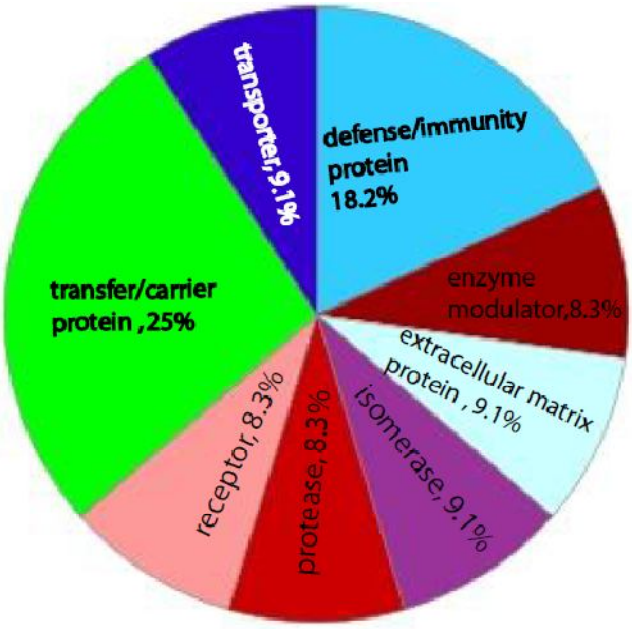


**Figure 8**. Summary of all differentailly expressed proteins in active and inactive LN urine. These significantly differed protein were classified based on their molcular functions.

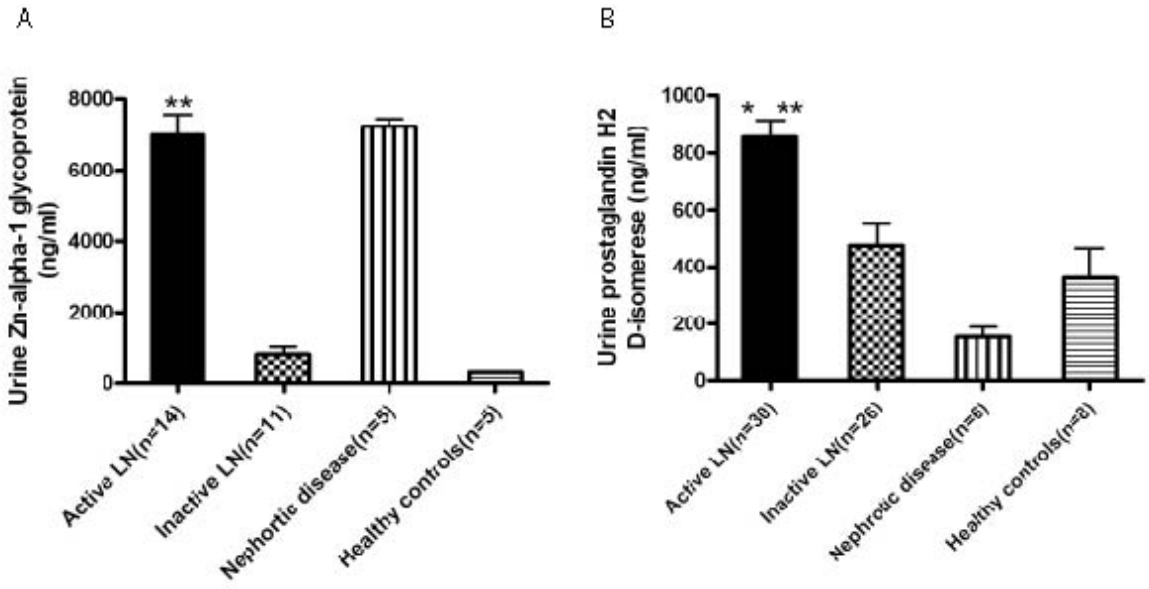


**Figure 9**. Urinary excretion of ZA2G and PGDS. Values are mean +/- SEM. Signifiicant differences are based on Tukey post doc testing. The histograms show level ZA2G (A) and PGDS (B) for active LN, inactive LN, healthy controls and nephrotic diseases. Significant differences between groups are indicated as follows: * = $p<0.002$, **=$p<0.0004$

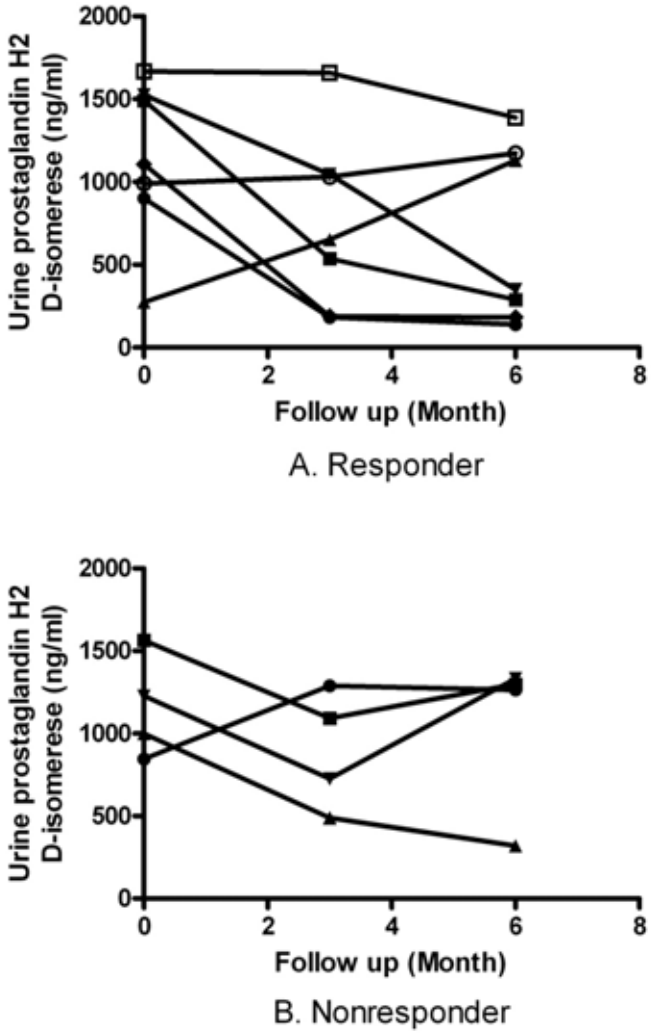


**Figure 10**. Urinary excretion of PGDS follow up treatment 0, 3 and 6 month. A) responder and B) nonresponder.

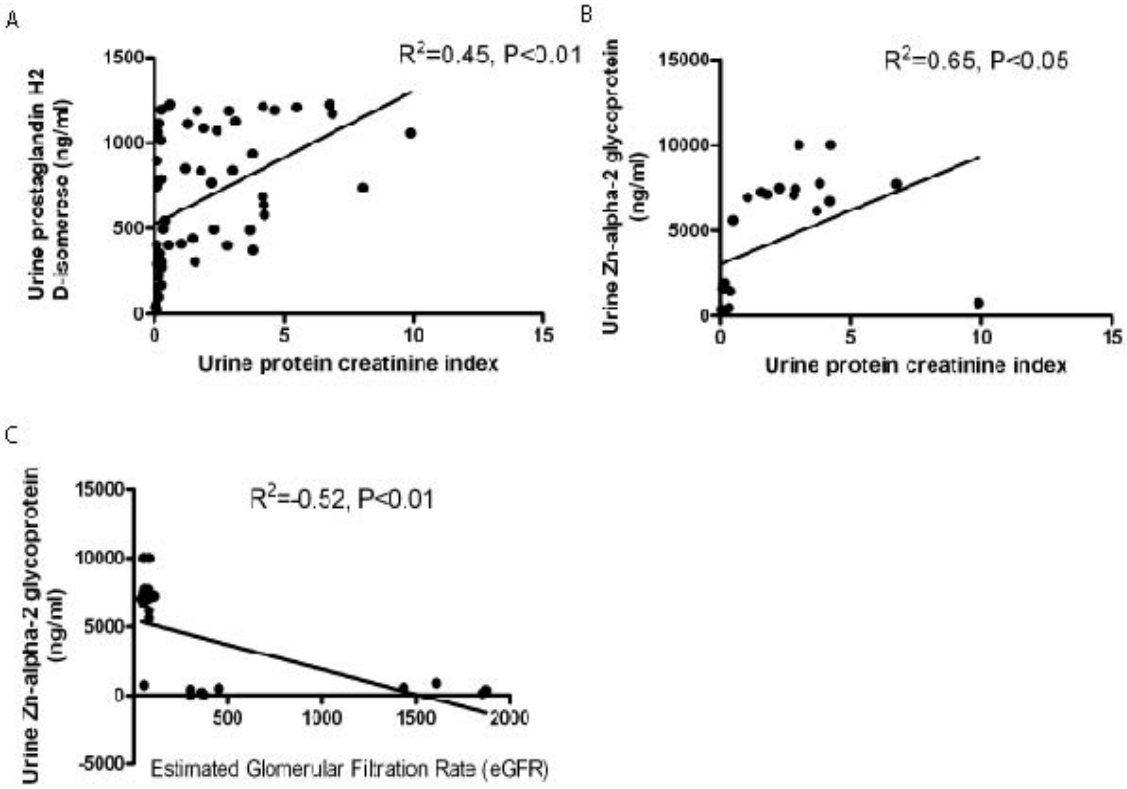


**Figure 11**. Correlation between PGDS, ZA2G and UPCI, eGFR. The relationship between PGDS (A), ZA2G (B) and UPCI were significant $P<0.01(R^2=0.45)$, $P<0.05(R^2=0.65)$, respectively. (C) The relationship between was significant $P<0.01(R^2=-0.52)$

### 1.2.1 Response and nonresponse Patient's characteristics

For 2-DE in group 2, six patients were enrolled in this time. A total of 6 patients were treated with Mycophenolate mofetil. After 6 months of mycophenolate therapy, 3 patients responded to treatment (responders) and 3 patients did not (nonresponders). The responder and nonresponder groups were generally comparable in age, disease activity, serum creatinine, estimated glomerular filtration rates, serum albumin, urine protein creatinine ratio, exposure to steroid, SLEDAI score and renal pathological indices (Table 9).

### 1.2.2 Proteome Map of urine in LN patients

The identification of protein had been performed with the separation of the protein extract from responder and non-responder though pH 3-10L, 7cm at the first dimension. One hundred and fifty microgram proteins were used in dependent sample and separated proteins were visualized with coomassie blue as shown in figure 12. About 200 spots were visualized after staining. Twenty-six protein spots in responder LN gel as reference for matching were excised and subjected to ESI/MS/MS (figure 13). Locations of all these identified proteins on 2-D gels were showed their accession number from SWISSPROT, percent of sequence coverage, theoretical isoelectric points (pI), and molecular weights (Mw) as summarized in Table 9

### 1.1.3 Differentially expressed spots

Relative volume of each spot was compared and analyzed of the paired proteome profiles between responder and non-responder by using ImageMaster software. We found that 12 were spots differentially expressed and 2 spots were present and absent. As Figure

14 shown that relative volume spot that had significantly differential expressed between responder and non-responder. In the table10 show lists of protein that significant expression.

We classified all differentially expressed protein into protein functional group on the basis of their biological process, including 1) cell communication; alpha-β1-glycoprotein 2) cellular companent organization; gelsolin 3) cellular process; alpha-β1-glycoprotein 4) metabolic process; protein AMBP 4) response to stimuli; alpha-β1-glycoprotein, protein AMBP, Zinc-α2-glycoprotein, Ig-gamma-2-chain C region 5) transport; serum albumin 6) developmental process; gelsolin and 7) immune system process; alpha-β1-glycoprotein, protein AMBP, Zinc-α2-glycoprotein, Ig-gamma-2-chain C region, CD59 as shown in figure 14

**Table 9** Comparisons between responder and nonresponder groups in demographics, laboratory results, and treatments received during the study

| Patient characteristics | Responders (n=3) | Nonresponders (n=3) |
|---|---|---|
| Sex, female: male | 3:0 | 3:0 |
| Age, years | 30.00±4.16 | 34.67±2.86 |
| eGFR (MDRD) (ml/min) | 94.03±22.36 | 83.24±10.35 |
| Pre-treatment Urine protein creatinine ratio (g/day) | 0.10±0.06 | 2.10±0.30 |
| SLEDAI-2K | 1.33±1.33 | 18.00±2.00 |
| White blood cell(cell/hyperfield) | 4.66±0.88 | 13.33±3.53 |
| Red blood cell(cell/hyperfield) | 2.66±0.67 | 9.67±2.72 |
| ISN/RPS classification<br>Class III, n<br>Class IV, n | <br>2<br>1 | <br>1<br>2 |
| Chronicity index (0–12) | 2.33±0.33 | 2.33±0.88 |
| Activity index(0-12) | 9.00±3.21 | 14.00±1.53 |
| Dosage of steroid at baseline (mg/d) | 18.33±7.26 | 18.33±6.66 |
| Dosage of steroid at 6 months (mg/d) | 7.59±3.82 | 10.83±5.07 |
| Dosage of MMF (mg) | 1833±166.67 | 2166±166.67 |

Abbreviations; eGFR, estimated glomerular filtration rate; ISN/RPS, International Society of Nephrology/Renal Pathology Society; MDRD, Modification of Diet in Renal Disease; MMF, mycophenolate mofetil; SLEDAI, SLE disease activity index.

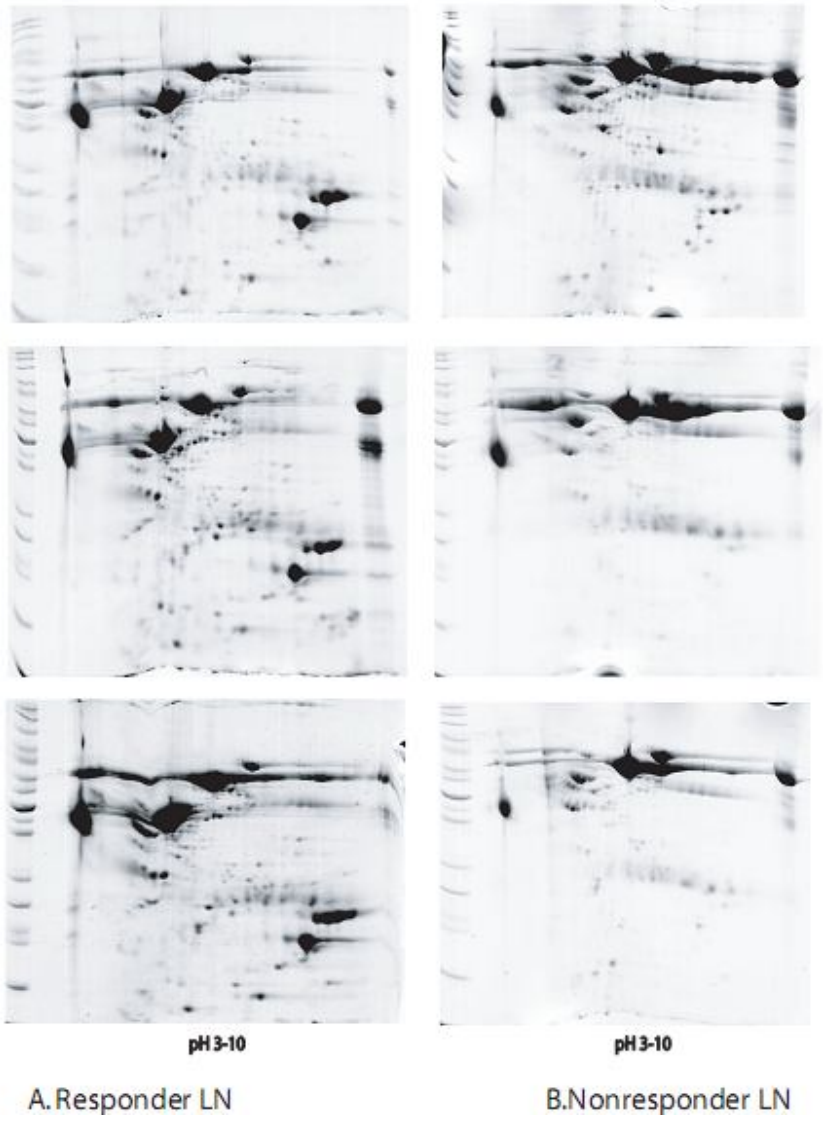


**Figure 12**. Two-DE gels from responder and nonresponder LN. Proteins were resolved using a linear pH 3–10 gradient in the first dimension and by SDS-PAGE on a 12.5% acrylamide gel in the second. Gels were stained with Coomassie blue. Patients were defined as responder LN (A) and nonresponder LN (B), respectively

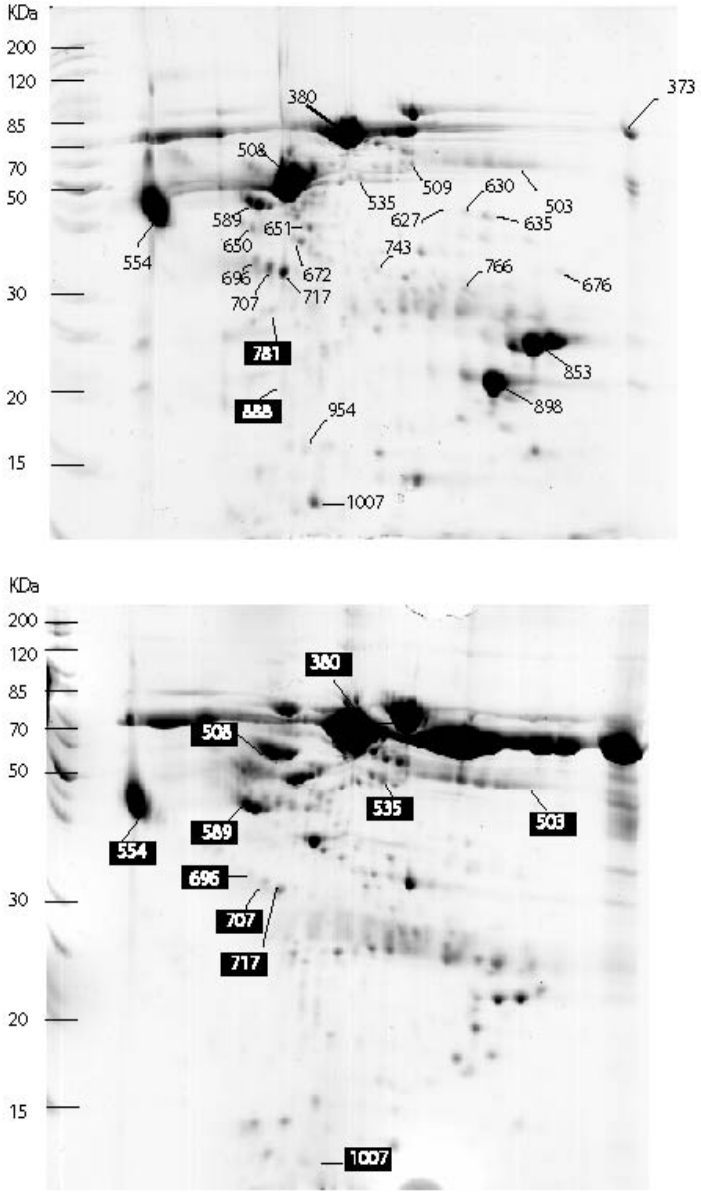


**Figure 13**. The reference 2-DE gels from responder (A) and nonresponder LN (B). Protein were resolved using a linear pH 3-10 gradient in the first dimensional and by SDS-PAGE on a 12.5% acrylamide gel in a second. Gels were stain with coomassie blue. Protein spots were identified by LC/MS/MS. (A) total 26 spots were identified in the active LN (summarized in the table2). Boxes in (B): Fourteen differentially expression spots by using Image Master 2D Platinum software (Amersham Bioscience) and student unpaired T-test.

### 1.2.4 Validation of protein expression

Urinary Protein AMBP and CD59 glycoprotein tend to increase in responder LN patients. We selected Protein AMBP, known as Alpha-1-microglobulin (A1M) and CD 59 glycoprotein for validation in an independent sample set because A1M and CD59 play a role in immunosuppressive protein. We found two proteins were significantly up-regulated in responder as compared with nonresponder (Figure 14) from 2-DE gels. We validated two proteins in an independent set of samples by using ELISA. At 6 month after treatment, urinary excretion of A1M and CD59 glycoprotein were no significant differences between responder and nonresponder groups as shown in figure 16.

Following to treatment at 6 month, we measured the change in urinary excretion of A1M and CD 59 glycoprotein in responder and nonresponder patients are shown as in figure 17 and 18, respectively. As compared to pretreatment, we found urinary excretion of A1M and CD59 glycoprotein did not have significant change at 6 month in both groups.

Table 10. The identified proteins from responder LN urine as reference

| No Match | Protein | Accession number | pI /MW | Score | Seq Cov% | No. of |
|---|---|---|---|---|---|---|
| 503 | Ig-gamma-2-chain C region | P01859 | 7.66/36.50 | 242 | 21 | 7 |
| 508 | Serum albumin | P02768 | 5.92/71.32 | 1777 | c | 66 |
| 535 | Serum albumin | P02768 | 5.92/71.32 | 733 | 44 | 21 |
| 554 | Alpha-β1-glycoprotein | | 5.56/54.79 | 458 | 34 | |
| 589 | Zinc-α2-glycoprotein | P25311 | 5.71/34.47 | 500 | 53 | 22 |
| 627 | Ig-gamma-2-chain C region | P01859 | 7.66/36.51 | 111 | 20 | 8 |
| 630 | Ig-gamma-2-chain C region | P01859 | 7.66/36.51 | 223 | 37 | 20 |
| 635 | Ig-gamma-1-chain C region | P01859 | 8.46/36.60 | 195 | 25 | 15 |
| 650 | Alpha-1-antitrypsin | P01009 | 5.37/46.88 | 137 | 22 | 10 |
| 651 | Serum albumin | P02768 | 5.92/71.32 | 738 | 50 | 47 |
| 672 | Serum albumin | P02768 | 5.92/71.32 | 742 | 38 | 48 |
| 676 | Ig-gamma-2-chain C region | P01859 | 7.66/36.51 | 96 | 32 | 10 |
| 696 | Protein AMBP | P02760 | 5.95/39.87 | 239 | 25 | 19 |
| 707 | Protein AMBP | P02760 | 5.95/39.87 | 432 | 26 | 23 |
| 717 | Protein AMBP | P02760 | 5.95/39.87 | 789 | 25 | 23 |
| 740 | Ig kappa chain C region | P01834 | 5.85/11.77 | 73 | 35 | 2 |
| 743 | Ig-gamma-2-chain C region | P01859 | 7.66/36.51 | 156 | 45 | 5 |
| 766 | Immunoglobulin lambda like Polypeptide 5 | B9A064 | 9.08/23.35 | 209 | 35 | 8 |
| 781 | Gelsolin | P06396 | 5.90/86.40 | 85 | 2 | 2 |
| 800 | Serum albumin | P02768 | 5.92/71.32 | 513 | 40 | 33 |
| 828 | Serum albumin | P02768 | 5.92/71.32 | 336 | 26 | 22 |
| 853 | Serum albumin | P02768 | 5.92/71.32 | 1625 | 21 | 77 |
| 856 | Alpha-1-antitrypsin | P01009 | 5.37/46.88 | 106 | 6 | 3 |
| 879 | Protein AMBP | P02760 | 5.95/39.87 | 178 | 23 | 11 |
| 888 | CD59 glycoprotein | P13987 | 6.02/14.67 | 55 | 25 | 4 |
| 942 | AP20 region protein 1 | Q81VJ8 | 10.09/18.74 | 26 | 3 | 1 |

Table 11. Differently expressed protein between responder and nonrepsonder group

| spot No | Protein ID | Accession Number | pI/MW (KDa) | Protein score | % Seq Cov | % Vol relative(arbitrary unit) Responder (Mean±SEM) | Nonresponder (Mean±SEM) | ratio N/R | p -value |
|---|---|---|---|---|---|---|---|---|---|
| **Plasma proteins** | | | | | | | | | |
| 380 | Albumin | P02768 | 5.92/71.73 | 1426 | 57 | 26.68±4.69 | 65.20±3.08 | 2.44 | 0.002 |
| 503 | Ig-gamma-2 chain region C | P01859 | 7.66/32 | 194 | 32 | 0.09±0.05 | 0.23±0.02 | 2.56 | 0.05 |
| 508 | Albumin | P02768 | 5.92/71.73 | 1020 | 48 | 22.76±0.44 | 3.40±0.79 | 0.15 | 0.001 |
| 535 | Albumin | P02768 | 5.92/71.73 | 659 | 43 | 0.14±0.02 | 0.03±0.01 | 0.21 | 0.009 |
| 554 | Alpha-beta1 glycoprotein | P04217 | 5.56/54.79 | 458 | 34 | 11.52±1.32 | 4.39±0.43 | 0.38 | 0.075 |
| 589 | Zing-alpha2 glycoprotein | P25311 | 5.71/34.63 | 450 | 53 | 2.14±0.16 | 4.39±0.43 | 2.05 | 0.069 |
| 853 | Albumin | P02768 | 5.92/71.73 | 1494 | 21 | 8.99±1.46 | 0.12±0.05 | 0.01 | 0.004 |
| 1007 | Albumin | P02768 | 5.92/71.73 | 645 | 37 | 0.32±0.05 | 0.04±0.007 | 0.13 | 0.005 |
| **Protein Binding** | | | | | | | | | |
| 696 | Protein AMBP | P02760 | 5.95/39.88 | 233 | 25 | 0.18±0.06 | 0.02±0.008 | 0.11 | 0.023 |
| 707 | Protein AMBP | P02760 | 5.95/39.88 | 422 | 25 | 0.30±0.008 | 0.06±0.014 | 0.20 | 0.001 |
| 717 | Protein AMBP | P02760 | 5.95/39.88 | 355 | 25 | 0.37±0.02 | 0.07±0.01 | 0.20 | 0.004 |
| **Receptor Biding** | | | | | | | | | |
| 888 | CD59 | P13987 | 6.02/14.80 | 52 | 25 | 0.03±0.009 | 0.00±0.00 DIV/0 | - | |
| **Calcium ion biding** | | | | | | | | | |
| 781 | Gelsolin | P06396 | 4.85/31.04 | 85 | 4 | 0.13±0.05 | 0.00±0.00 DIV/0 | - | |

% volume relative; [(volume of spot/volume over all the spots in the gel)x100], pI/MW; isoelectric point/molecular weight, R/N; Response LN/ Non-response LN, KDa; kilodalton, DIV; Divived by zero

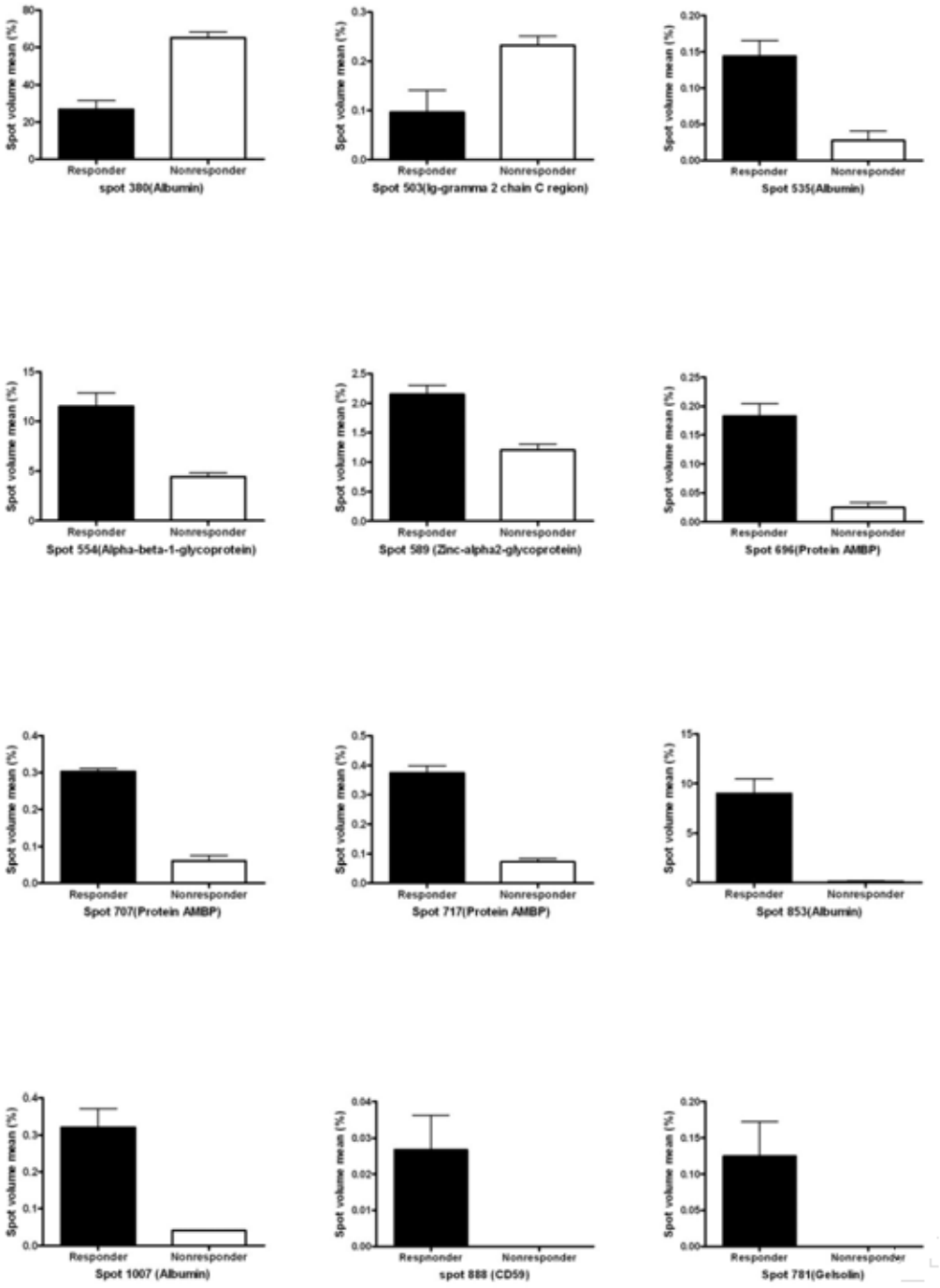


**Figure 1**4. Histogram represents of the relative volume mean (%) of 12 spots that had significantly differential expression. Value of plotted are mean +/- SEM.

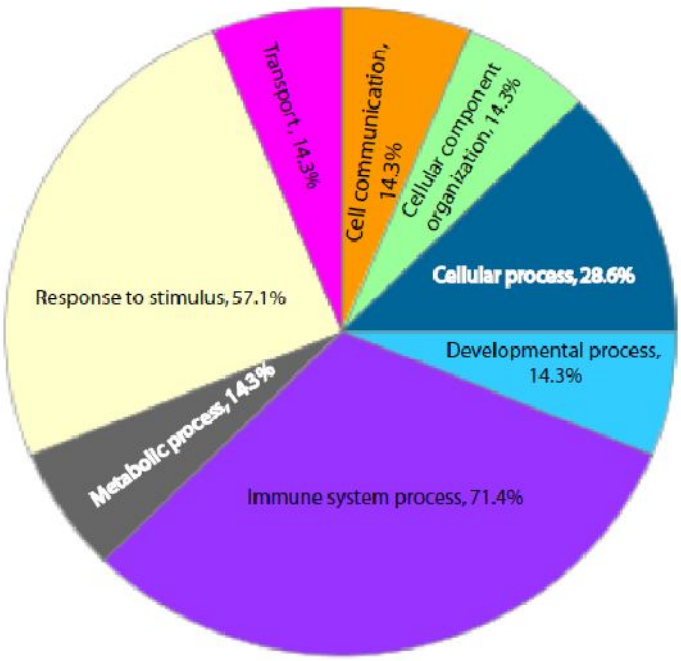


**Figure 15**. Summary of all differentailly expressed proteins in reponder and nonreponder LN urine. These significantly differed protein were classified based on their biological process.

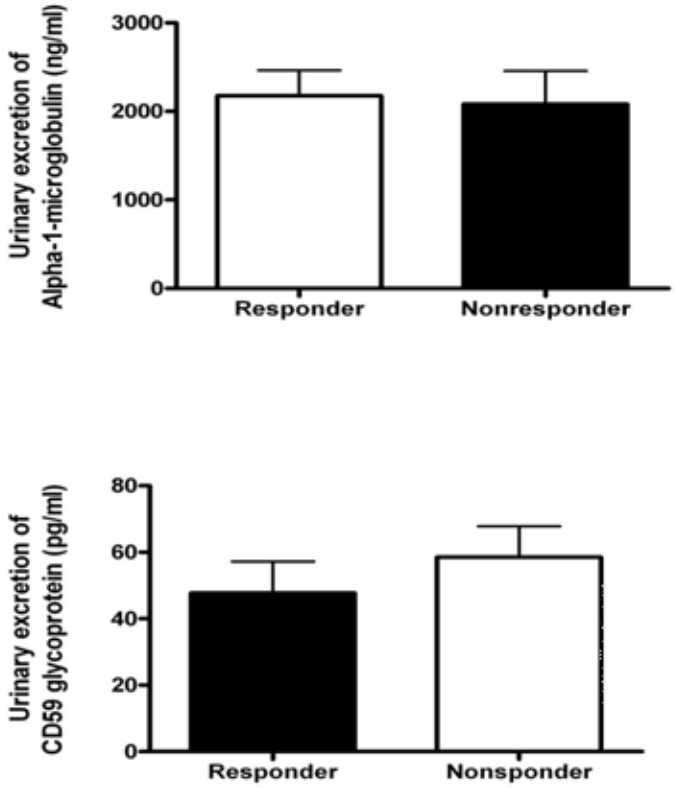


**Figure 16**. Urinary excretion of A1M and CD 59 glycoprotein. Values are mean +/- SEM. The histogram show level A1M (above) and CD59 glycoprotein (below) for responder and nonresponder.

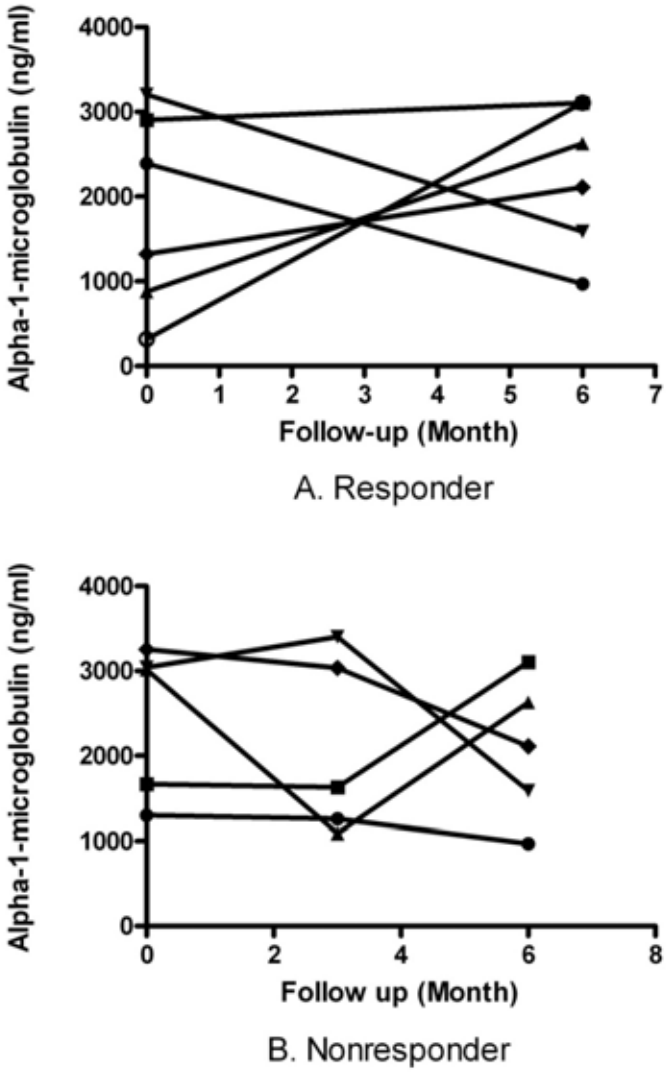


**Figure** 17. Change in urinary excretion of A1M in responder LN (n=6) and nonresponder LN (n=5) with 0, 3 and 6 month.

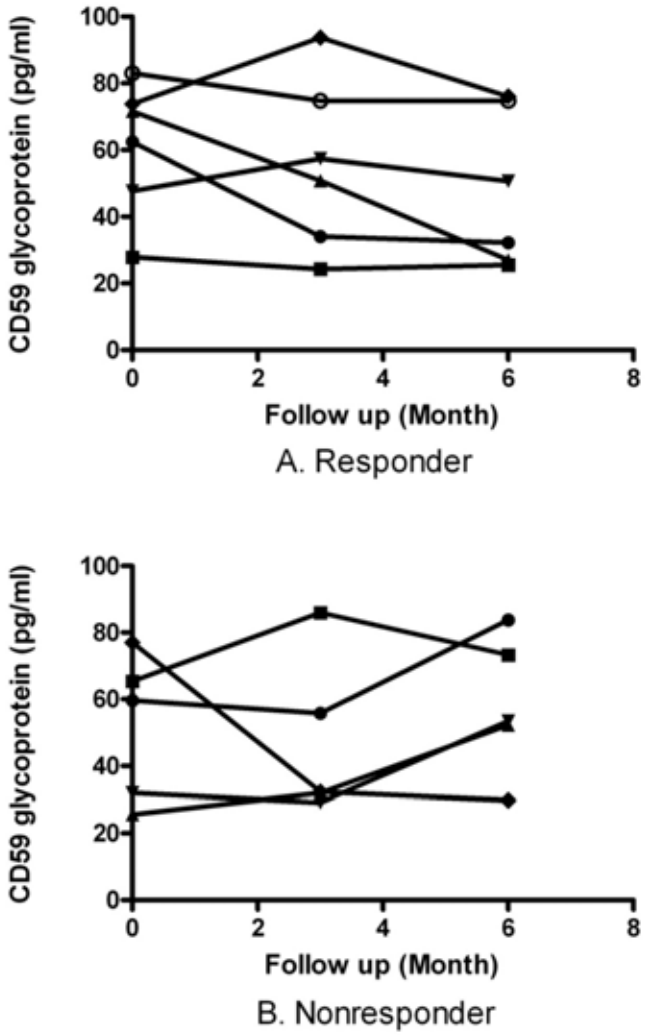


**Figure 18**. Change in urinary excretion of CD 59 glycoprotein in responder LN (n=6) and nonresponder LN (n=5) with 0, 3 and 6 month.

# CHAPTER V

# DISCUSSION AND CONCLUSION

The non-invasive biomarkers are needed for determining the LN disease activity due to the possibility of post-renal biopsy complication, especially in LN patients with frequent relapse. In this study, we used proteomics approach for urinary biomarkers discovery in active lupus nephritis.

Several techniques of urine proteomic studies either with gel-base or gel-free proteomic analysis such as LC/MS/MS (Liquid chromatography tandem mass spectrometry), 2-DE with MALDI/TOF/MS (Matrix-assisted laser desorption/ionization/mass spectrometry) or, a newer technique, SELDI-TOF/MS (Surface-enhanced laser desorption/ionization time of flight mass spectrometry). Recently, several candidate biomarker proteins such as hepcidine, prostaglandin D synthase (PGDS), alpha-1-acid-glycoprotein, transferring and ceruloplasmin were discovered by SELDI-TOF approach (Suzuki, Wiers, 2009, Zhang, Jin, 2008) . However, regardless of the proteomic techniques for the protein discovery, further validation in a large sample size is essential.

Then, the 2-DE proteomic approach with the different protein spots identification by ESI/MS/MS was chosen for our study. We found that the different protein expression between active and inactive LN could be arranged into the different groups according to the protein functions including; albumin, serotransferrin, Ig Kappa C and Ig kappa chain V-III HAH region, alpha-1-beta-glycoprotein, haptoglobin and beta-2-microglobulin.It is most likely that these plasma

proteins, mostly inflammatory mediators, were excreted into the active LN urine. Of these proteins we chose to validate 2 proteins in a larger, independent urine samples set.

ZA2G, a single chain polypeptide found in body fluids including urine, serum, cerebrospinal fluid, and saliva (Schwick and Haupt, 1981, Bundred, Miller, 1987, Jain, Rajput, 2005, Schmitt, Marlier, 2008), is associate with several functions including lipid mobilization, fertilization, repairing process and, possibly, immune response. The ZA2G knock-out mice had overweight due to ZA2G stimulates adiponectin secretion (Bing, Mracek, 2010). In addition, ZA2G is linked to the decreased cAMP level involve in the human sperm motility which affect the fertilization(Qu, Ying, 2007). Both in-vivo and in-vitro data confirmed that ZA2G inhibit tubular proliferative response and then delayed recovery of kidney damages (Schmitt, Marlier, 2008). ZA2G,similar structure with Class I Major histocompatibility complex (MHC) but not associate with $\beta_2$-M light-chain(Araki, Gejyo, 1988),is able to bind non-peptide compound and might associate with T cell lipid recognition (Sanchez, Chirino, 1999). Therefore, it is possible that ZA2G may have some function related with the activated immune response in LN patients. ZA2G, relatively large protein with 40 kDa, is believed to be filtrated through glomerular filtration but rarely excreted in urine of healthy people (Burgi and Schmid, 1961). We found that urinary ZA2G were increased non-specifically in active LN urine(Figure 3A) but also in other glomerular diseases urine as previously report(Jain, Rajput, 2005). Moreover, urinary ZA2G was found in urine of bladder cancer patients.(Irmak, Tilki, 2005)

However, the urinary ZA2G might be a result of the increase glomerular filtration and/or decrease tubular reabsorption and/or increase tubular secretion in the pathological conditions which seems not necessary to be an inflammatory process. We found urinary ZA2G in FSGS and minimal change disease which not show inflammatory urine sediment. We hypothesized that the mechanisms ZA2G urinary excretion was most likely the increase glomerular filtration regardless of the inflammatory process. Further studies will be required for answering this question.

Another candidate protein form our report was urinary PGDS. PGDS is a homodimer with each subunit ranging in size from 21-31 KDa, depending on its post-translational forms such as glycosylation form or lipocalin form (Urade and Hayaishi, 2000).PGDS catalyzes the isomerization of prostaglandin $H_2$ ($PGH_2$) to produce prostaglandin $D_2$ ($PGD_2$) which is involved in sleep regulation, platelet aggregation, allergic, inflammatory response and chemoattractant properties(Lewis, Soter, 1982, Urade, Ujihara, 1989). In the kidneys, PGDS is constitutively expressed in podocytes, mesangial cell, tubular epithelial cells of outer medulla and cortex in primate normal kidneys. Moreover, PGDS level was strongly correlated with severity of antiglomerular basement membrane induced nephritis (Wu, Fu)). PGDS is associated with the tubular epithelium apoptosis and glomerular inflammation in diabetes mice. L-PGDS was one of the diabetic biomarker candidates.

The level of prostaglandin D synthase (PGDS) was increased in the urine of active LN but not other glomerular disease (figure3B) as previously report in pediatric LN with SLEDI/MS/MS (Suzuki, Wiers, 2009).

Moreover, we studied other group that following for treatment for 6 months. Patients can be divided into 2 groups as responder and nonresponder. A little study

has used a longitudinal study for proteomics to identify specific proteins that can be predicted after treatment with immunosuppressive drug and validated in an independent a sample set.

We found that the different protein expression between groups could be arranged into the different groups according to the protein functions including; Plasma proteins: Ig gamma chain C region, alpha-β1-glycoprotein, and Zinc-α2-glycoprotein; Protein binding: protein AMBP; receptor binding: CD59 glycoprotein; Calcium binding: gelsolin.

CD59 glycoprotein is glycosyl-phosphatidylinositol(GPI)-anchor glycoprotein and has molecular weight about 18-20 KDa. CD59 binds to the α-subunit of C8 and C9b domain of C9 and than preventing formation of a lytic lesion by limiting incorporation of C9 into C5b-9 complex. In human kidney, CD59 is expressed in glomerular cell. There is evident shown that CD 59 has an important protective role in kidney damage. Masuto and co-worker demonstrated that neutralization of CD59 resulted in more severe glomerular damage in rat with experimental mesangial proliferative glomerulonephritis induced with a lectin. Moreover, CD59 knockout mice developed more severe glomerular histologic feature. Our data showed presence of CD59 after patient response to treatment. This result suggests that CD59 may an important role to protect in autoimmune tissue injury.

Protein AMBP is also known as alpha-1-microglobulin (A1M). A1M is a 27 kDa glycoprotein and secreted in various body fluids including serum and urine. A1M plays an immunoprotective role and is an anti-oxidant that can scavenge pro-oxidant heme groups. A1M is reabsorbed in urine by the megalin receptor in proximal tubular cells, where it is then catabolized. However, small amount is excreted in urine.

Elevate of A1M have also been studied as a biomarker for renal tubular disorders in various disorders, including heavy metal poisoning , hypertension , multiple myeloma, and type 2 diabetic. In this study, we found that A1M is unregulated in responder patients from 2-DE data. It may be a useful marker for following response to therapy

Furthermore, two candidate proteins need to validate in an independent sample set. These findings are consistent with other proteiomics study that found that A1M in LN patient. However, CD59 glycoprotein is the first study that found in proteomics technique. It will thus be important to characterize 2 proteins that are increased during treatment, suggesting it may be a useful marker for following response to therapy.

There were several limitation in our study, 1) hydroprobic protein and small molecular protein detection capacity in 2-DE is less sensitive. 2) The coomassie blue to visual a spot protein has low sensitivity, the low abundance protein might not be detected.

In conclusion, we used 2-DE proteomic studies to discover new active LN biomarker candidates with the independent patient set for validation and found that urinary PGDS might be a good biomarker for active LN diagnosis whereas ZA2G might became urinary biomarkers of general glomerular diseases including lupus.

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# APPENDIX

1. 2D Lysis Buffer solution

| | | |
|---|---|---|
| 1. | Urea | 4.2040 g |
| 2. | Thiourea | 1.5224 g |
| 3. | CHSPA | 0.4 g |
| 4. | DTT | 0.1851 g |
| 5. | Tris-base | 0.0485 g |
| 6. | Protease inhibitor | 10 mL |

Dissolve in DI water and make volume to 10 mL, keep at - 20°C.

2. Rehydration stock solution without IPG buffer

| | |
|---|---|
| 1. Urea | 4.2020 g |
| 2. Thiouea | 1.5224 g |
| 3. CHAPS | 0.20 g |
| 4. DTT | 0.1851 g |

Dissolve in DI water and make volume to 10 mL keep at -20°C.

3. SDS equilibration buffer

| | |
|---|---|
| 1. Tris –HCl | 10 mL |
| 2. Urea | 72.07 g |
| 3. Glycerol | 69 mL |
| 4. SDS | 4.0 g |
| 5. Bromophenol blue | 400 uL |

Dilluted in DI water and make volume to 200 uL keep at -20°C.

4. 4X resolving gel buffer

1. Tris-HCl 181.7 g

Dissolve in DI water and make volume to 750 mL adjust to pH 8.8 and adjust volume to 1 L

5. SDS electrophoresis buffer

| | |
|---|---|
| Tris-base | 30.3 g |
| Glycine | 144.0 g |
| SDS | 10.0 g |

Dissolve in DI water and make volume to 1 L.

## BIOGRAPHY


| | |
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