Predictive Biomarker Panel in Proliferative Lupus Nephritis, Two-Dimensional Shotgun Proteomics

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Introduction. Lupus nephritis (LN) is one of the most serious complications of systemic lupus erythematous (SLE). With no specific clinical or laboratory manifestation to predict response to treatment, this study was aimed to provide a panel of predictive biomarkers of response before initiation of treatment.
Methods. Liquid chromatography tandem mass spectrometry (LC-MS/MS) analysis was performed on plasma and urine samples of 11 patients with biopsy proven proliferative LN at the time of biopsy. Unsupervised principal component analysis (PCA), orthogonal projection to latent structures discriminant analysis (OPLS-DA), gene ontology annotation and protein mapping were performed on 326 proteins in plasma and 1381 proteins in urine samples.
Results. Samples of eight patients achieved complete remission and three reached partial remission were analyzed. The mean 24-hour protein excretion was 3259 mg/d and the mean eGFR was 87.73 cc/min. OPLS-DA analysis of plasma samples showed a clear discrimination for complete and partial remission patients. Twenty plasma proteins and ten urine proteins with the highest fold changes and AUCs were selected as candidate biomarkers (IGHV1-18, PI16, IGHD, C3, FCER2, EPS8L2, CTTN, BLVRB). This plasma and urine biomarker panel is involved in oxidative stress, acute inflammation, reduction in regulatory T cells, complement pathway consumption, and proximal tubule bicarbonate reclamation.
Conclusion. Our suggested panel of plasma and urine biomarkers can precisely discriminate patients with possibility of complete response to treatment. It seems that the higher indices of inflammation will associate with better chance of achieving complete remission.

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INTRODUCTION

SLE is an autoimmune multisystem disease. Up to 80% of patients with SLE will develop LN, a serious complication which may lead to end stage kidney disease and is associated with poor survival.[1] Diagnosed by kidney biopsy, the mainstay of treatment in proliferative LN is immunosuppressive therapy. Various clinical and laboratory characteristics were proposed as predictive markers of responsiveness to treatment at the 6th month, such as baseline C4 level, estimated glomerular filtration rate (eGFR), reduction in proteinuria,[2] and increase in serum albumin level after 3 months.[3] Waiting six months after initiation of induction therapy to evaluate whether the desired response is achieved or not, as recommended by American College of Rheumatology (ACR), may result in irreversible

kidney damage. Biomarkers may help to predict response to treatment at induction and may guide physicians to change treatment earlier if proper response is not detected. Furthermore, they may help to choose specific immunosuppressive drugs with higher chance of response. In order to introduce a panel of biomarkers which may predict response to therapy among patients with proliferative LN, we performed proteomics study on the plasma and urine samples of patients with biopsy proven class III or IV LN at the time of kidney biopsy, and followed them for up to 4 years.

MATERIALS AND METHODS

Patients

Nineteen patients (3 males and 16 females) with biopsy-proven LN were consecutively enrolled in this study during 2015 and 2016, at Labbafinejad Hospital. Pathology samples were reviewed by a single pathologist and were reported based on 2003 International Society of Nephrology (ISN) and the Renal Pathology Society (RPS). Demographic characteristics of the patients, laboratory findings, eGFR based on CKD-EPI equation, and the amount of proteinuria at the time of biopsy were recorded. Patients with co-morbidities like diabetes, infections and malignancies, and gross hematuria were excluded. Urine and plasma samples were collected on the day of biopsy. Informed consents were obtained from all patients according to consent form of "Medical Ethics" committee of Shahid Beheshti University of Medical Sciences. Patients were followed for up to 4 years, and response to treatment was evaluated. Remission was defined according to the following criteria:[4] Complete response as urine protein < 500 mg/24h plus (near) normal renal function, and partial response as more than 50% decrease in proteinuria, to at least sub-nephrotic levels plus stabilization of serum creatinine levels.

Samples Collection and Preparation

Plasma. Five mL blood was collected from patients in tubes containing K_2EDTA.plasma was separated carefully from the sediment after centrifugation at 4000 rpm for 8 minutes at 4°C. Protease inhibitor was added to plasma and stored at -80°C.

Urine. Approximately 20 to 40 mL of second morning midstream urine were collected and treated with protease inhibitor cocktail (Sigma). Samples were concentrated and desalted by ultrafiltration following Kalantari *et al*.[5] and stored at −80°C for further processing.

Protein Assay and Reduction/Alkylation

Sample preparation protocol was similar to Saei *et al*.[6] The protein concentration in plasma and urine samples was measured using Pierce BCA Protein Assay (Thermo Scientific). Fifty µg of proteins were reduced and alkylated with 15 mM dithiothreitol and 20 mM iodoacetamide, respectively.

Protein Precipitation and Digestion

Proteins were precipitated by sequentially adding methanol, chloroform and water (4:1:3 v/v/v) to each sample and centrifuging at 21,000 g for 10 min. Each protein pellet was dissolved in 20 mM EPPS buffer (pH 8.5) containing 8 M urea and then digested with Lysyl endopeptidase LysC (Wako Pure Chemical Industries) and trypsin (Promega) at a ratio of 1:50 enzyme:protein.

TMT10plex Mass Tag Labeling

Samples were labeled with adding 10 µL of TMT10plex reagents dissolved in 40 µL of anhydrous acetonitrile (Thermo, Catalog Number: 90110) and incubated for 2h at room temperature. Each set of 10 samples were pooled, cleaned and desalted using Sep-Pak C18 (Waters). The samples were dried in Speedvac and stored at −80°C.

High pH Reversed-Phase Chromatography

Labeled peptides were fractionated into eight fractions using pierce high pH reversed-phase peptide fractionation Kit (Thermo) according to the manufacture's protocol (Thermo, Catalog Number: 84868). Fractions were dried using SpeedVac concentrator and stored at −80°C prior to LC-MS/MS analysis.

LC-MS/MS Analysis

One micrograms of labeled peptides were loaded onto a 50 cm EASY-Spray column (75 µm internal diameter, packed with PepMap C18, 2 µm beads, 100 Å pore size) connected to a nano flow Dionex UltiMate 3000 UPLC system (Thermo). Mass spectra were acquired with an orbitrap elite hybrid mass spectrometer (Thermo) in data-dependent mode at a nominal MS resolution of 120,000. From each

survey mass spectrum, 10 most abundant precursor ions were selected for MS/MS and isolated in a quadrupole with a 1.0 m/z unit window. The MS/MS fragments were detected with a nominal resolution of 30,000 in the m/z range from 375 to 1,200.

Data Processing

MaxQuant software version 1.5.6.5 was used for searching raw data.[7,8] The MS/MS data were searched using Andromeda search engine.[9] Search parameters was set as follow: Mass tolerance for precursor ions was 20 ppm (initial search) and 4.5 ppm (main search) and the MS/MS mass tolerance was set at 20 ppm. Cysteine carbamidomethylation was used as a fixed modification, while methionine oxidation and N, Q-deamidation were selected as variable modifications. Trypsin/P was selected as enzyme specificity. No more than two missed cleavages were allowed. A 1% false discovery rate was used as a filter at both protein and peptide levels. Reporter ion MS2 and 10plex TMT were selected as parameters for labeled raw files. For all other parameters, the default settings were used. After removing all the contaminants, only proteins with at least two peptides were included in the final dataset.

Statistical Analysis

The quantitative proteome data was subjected to multivariate statistical analysis using SIMCA (SIMCA 15, Umetrics, Umea, Sweden). Unsupervised principal component analysis (PCA) was performed without consideration of group information for observing the overview of the data structure. The subset of proteins with the highest potential to separate individuals based on their follow-up condition were identified with orthogonal partial least squares-discriminant analysis (OPLS-DA), and then protein selection was performed using the combination of Variable Influence in Projection (VIP) more than 1 and p (corr) more than 0.5.[10]

Gene Ontology Annotation

Gene ontology annotation and network analyses was performed by mapping the proteins to the PANTHER Classification System version 15.0,[11] STRING aversion 11.0[12] and DAVID version 6.8.

RESULTS

Clinical and Pathological Characteristics of Patients

Nineteen patients with biopsy-proven LN (mean age 33.9 years) were included in this study. Two patients with End-Stage Renal Disease (ESRD, interstitial fibrosis and tubular atrophy > 70%) and one dead patient were excluded. Based on ISN/RPS 2003, class III, Class IV and Class V were diagnosed in 1, 13, and 2 of cases, respectively. Among patients with proliferative LN (class III and IV), 11 patients completed the follow up period. All the patients were treated with prednisolone, Mycophenolate Mofetil/Sodium (MMF), and hydroxychloroquine as the initial treatment. In two patients in partial response group, Tacrolimus was added to induce remission after failing to achieve complete remission. Three patients were treated with Rituximab in order to induce complete remission, and successful remission achieved in

Table 1. Clinical, Laboratory, and Pathologic Characteristics of Patients with Proliferative Lupus Nephritis

Patient	Age (year)	Gender	eGFR* (cc/min)	Proteinuria (mg/d)	Activity Index (0 to 24)	Chronicity Index (0 to 12)	IF/TA# (%)	Response to Treatment
1	29	Female	76	3400	10	2	15	Complete
2	30	Female	116.3	2200	10	1	5	Complete
3	40	Female	92.2	7650	8	2	10	Complete
4	25	Female	78.2	1615	7	1	15	Complete
5	69	Female	62.5	4000	9	1	10	Complete
6	17	Female	132.6	3480	7	1	10	Partial
7	30	Female	98.9	1175	8	2	5	Complete
8	29	Male	53.3	4830	8	1	10	Partial
9	42	Female	67.8	2800	11	1	5	Complete
10	30	Female	85.8	2150	9	2	10	Partial
11	26	Female	101.8	2550	7	1	5	Complete

*eGFR: estimated glomerular filtration rate
#IF/TA: interstitial fibrosis and tubular atrophy

two of them.

Eight patients achieved complete remission and three reached partial remission. The mean 24 h protein excretion was 3259 mg/d and the mean eGFR was 87.73 cc/min. The mean activity index was 8.5 and chronicity index was 1.36. Mean of interstitial fibrosis and tubular atrophy was 9% (Table 1).

Plasma

Unsupervised Statistical Analysis by PCA. A total of 326 unique proteins were identified and quantified by LC-MS/MS in plasma samples. PCA score plot showed that patients with partial remission separate from the other patients especially from patients with completed remission (Figure 1A). This indicates the large differences between the

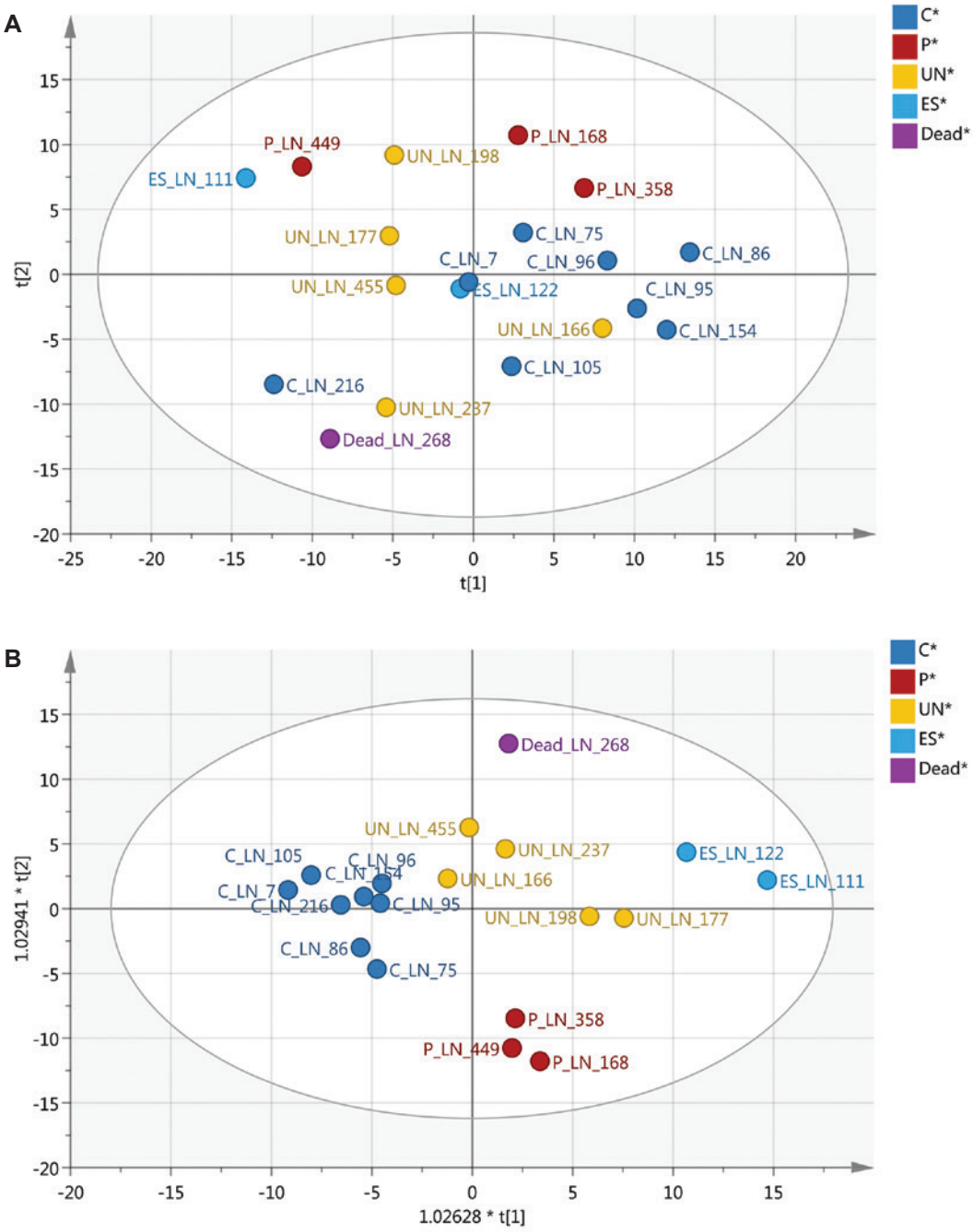


Figure 1. A) Unsupervised PCA Scores Plot Based on 326 Quantified Proteins Across All 19 Plasma Samples, B) OPLS-DA Scores Plot Based on 326 Quantified Proteins Across All 19 Plasma Samples (Abbreviations: LN, Lupus Nephritis; C, completed remission; P, partial remission; UN, unknown; ES, end-stage).

protein profiles of patients with partial and complete remission. There were no outliers on PCA score plots, which means that no confounding factor affected our study.

Supervised Statistical Analysis. OPLS-DA model was created for all 19 LN patients. OPLS-DA scores plot (Figure 1B) visualizing the separation of the subjects, showed a clear separation between groups. All patients in different groups clustered together. The clustering illustrated in Figure 1B indicates the large difference between the patient plasma protein profiles.

Variable Selection. OPLS-DA model could clearly separate complete and partial remission patients (Figure 2A) indicating a large difference in plasma protein profiles between two groups. Variable

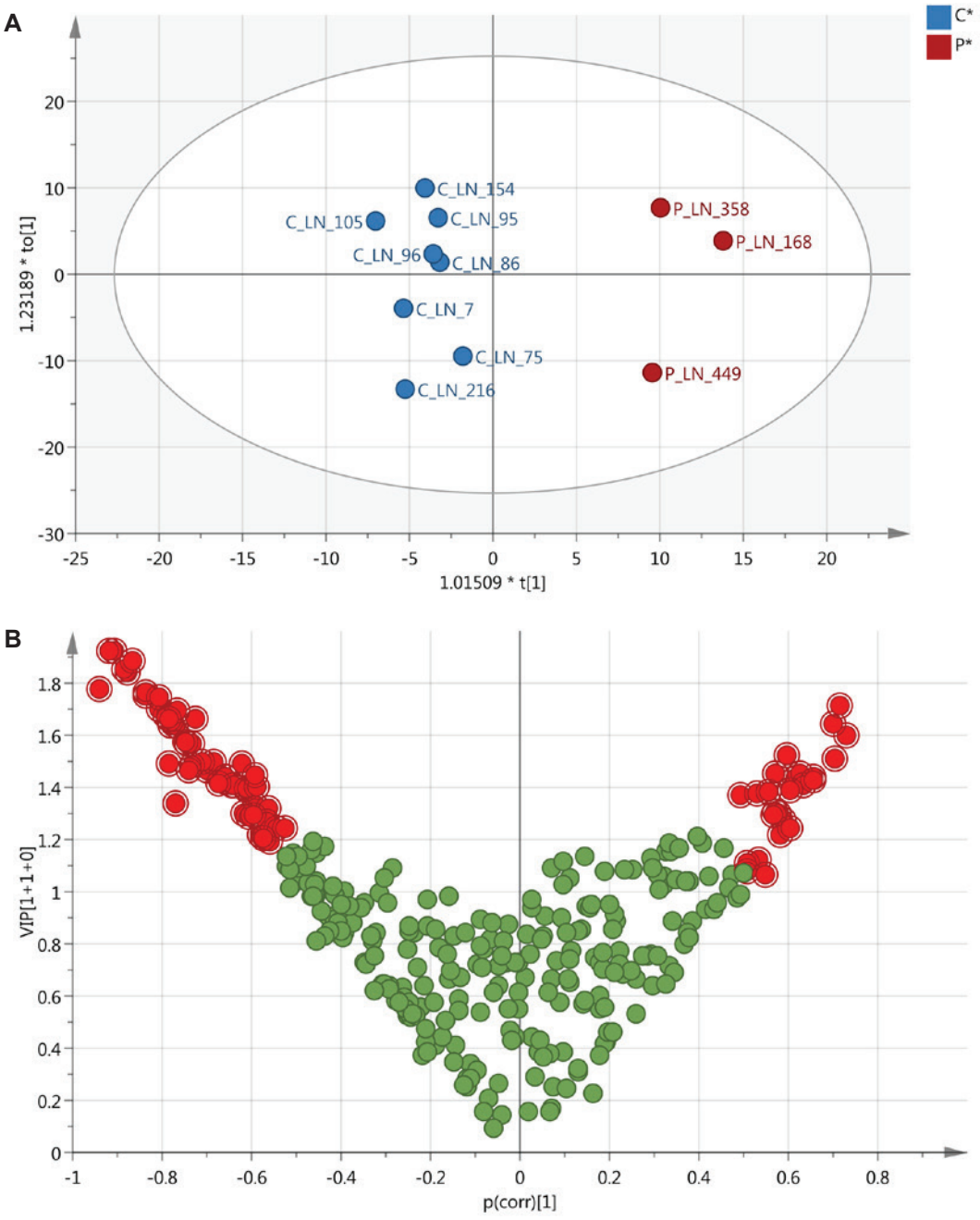


Figure 2. A) OPLS-DA Score Plot for Two Groups of Patients by Plasma Samples (Abbreviations: C, complete remission; P, partial remission) Used for Variable Selection, B) Variable Selection in Plasma Samples Using the Combination of Variable Influence in Projection (VIP) and P (corr), Using VIP > 1.0 and |p(corr)| > 0.5 as Inclusion Criteria.

selection (Figure 2B) showed 79 differential proteins of which 55 and 24 proteins had a significantly higher abundance in patients with complete and partial remission respectively. Twenty proteins with the highest fold changes and AUCs were identified as candidate biomarkers (Table 2).

Gene Ontology Analysis. Gene ontology enrichment analysis (Figure 3) based on molecular function revealed that most of 79 differentially represented proteins were involved in "binding" (59.5%). Many proteins mapped to the biological process of "response to stimulus" (15%). Based on cellular component, most of these proteins originated from "extracellular region" (39.2%).

STRING protein network analysis of 20 candidate biomarkers showed that 11 proteins had strong interactions in a significant network. No interaction was found for two other proteins (Figure 4). Pathway analysis according to KEGG database revealed that the majority of candidate biomarkers were involved in "complement pathway".

Urine

Unsupervised Statistical Analysis by PCA. A total of 1,381 unique proteins were identified and quantified by LC-MS/MS in urine samples. Score plot (Figure 5A) showed that there was no clustering among patients based on their urine protein profiles. This implies the relatively small differences between the patient urine proteome profiles.

Supervised Statistical Analysis. OPLS-DA scores plot of all patients showed clusters for two patients with partial remission separating them from other patients. Two end-stage cases clustered together, while the dead case was not separated from the others. All the other subjects clustered together (Figure 5B).

Variable Selection. OPLS-DA score plot (Figure 5C) showed a clear discrimination between two groups of partial and complete remission and variable selection was performed. In total, 278 proteins significantly contributed to the discrimination of subjects with complete versus partial remission, of which 118 and 160 proteins were significantly over-represented in patients with complete and partial remission respectively. Ten proteins with the highest fold changes and AUCs were selected as candidate biomarkers (Table 3).

Gene Ontology Analysis. Gene ontology enrichment analysis of 278 differential proteins (Supplementary File) showed "binding" (39.2%) and "catalytic activity" (37.4%) as the major

Table 2. Differentially Expressed Plasma Proteins in Lupus Nephritis Patients with Complete Remission Compared to Partial Remission

Protein Names	Gene Names	Fold Change*	Change Direction	AUC**
Immunoglobulin Heavy Variable 1-18	IGHV1-18	4.98	Up	1
Ig Kappa Chain V-I Region AU		4.72	Up	1
Peptidase Inhibitor 16	PI16	2.18	Down	1
Ig Delta Chain C Region	IGHD	7.71	Down	0.96
Complement C3	C3	2.93	Down	0.96
Haptoglobin-related Protein	HPR	2.55	Down	0.96
Complement Factor H-related Protein 5	CFHR5	3.27	Down	0.95
Immunoglobulin Lambda Variable 3-12	IGLV3-12	4.74	Down	0.92
Immunoglobulin Heavy Variable 4-4	IGHV4-4	3.24	Up	0.91
Ig Lambda Chain V-IV Region Bau		30.17	Up	0.87
Prenylcysteine Oxidase 1	PCYOX1	3.84	Down	0.87
Glutathione Peroxidase	GPX3	3.09	Up	0.87
Ig Alpha-2 Chain C Region	IGHA2	2.09	Down	0.87
C-reactive Protein (1 to 205)	CRP	5.96	Up	0.79
Catalase	CAT	3.09	Up	0.79
Mannose-binding Protein C	MBL2	2.98	Down	0.79
Serum Amyloid A-1 Protein	SAA1	7.33	Up	0.75
Lipopolysaccharide-binding Protein	LBP	2.98	Up	0.75
Filaggrin-2	FLG2	2.98	Up	0.64
Vitamin D-binding Protein	GC	57.97	Down	0.62

*Fold Change Based on Peak Intensity
**AUC: area under curve

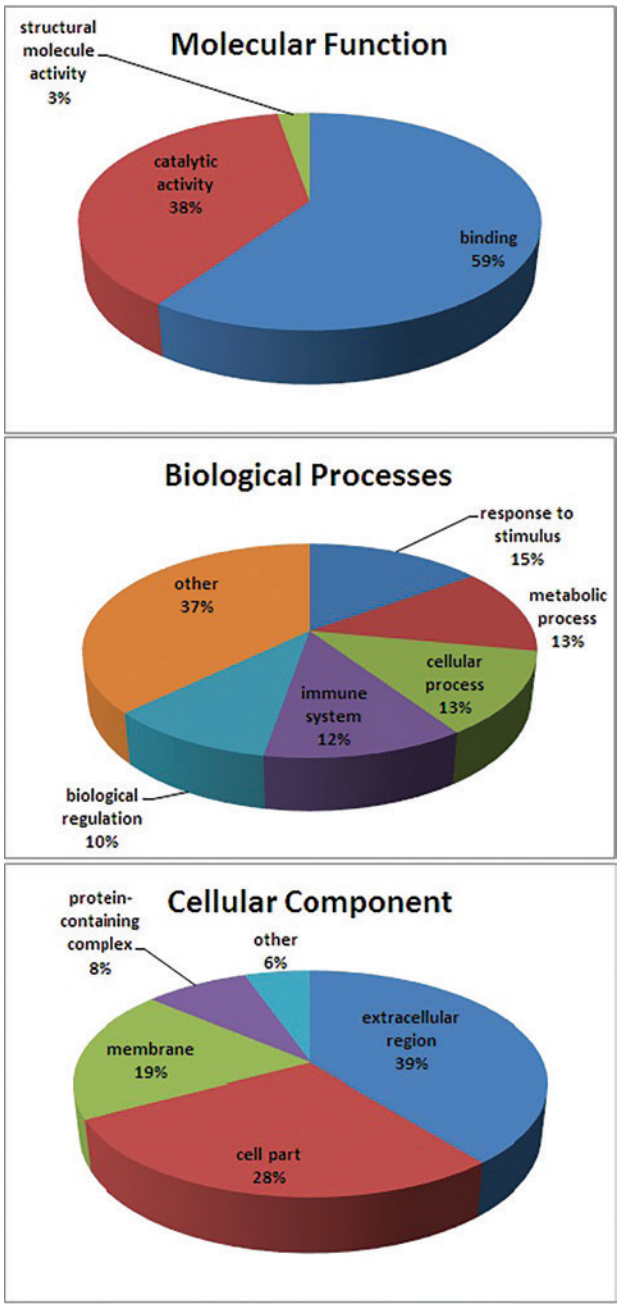


Figure 3. Gene Ontology Enrichment Analysis of 79 Most Important Proteins in Plasma

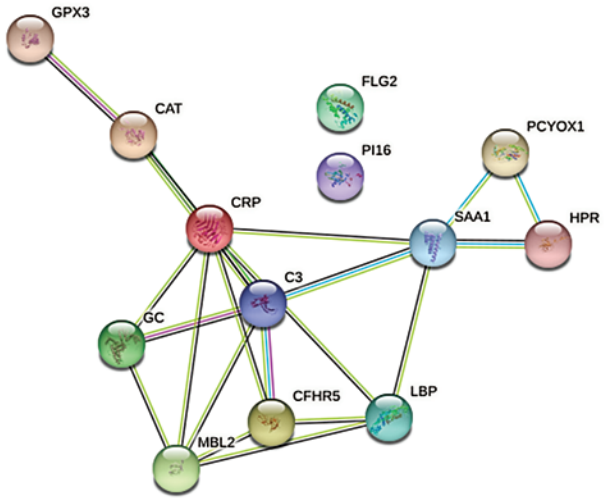


Figure 4. STRING Network Analysis of 20 Most Important Up/down-regulated Proteins in Plasma (13 proteins were identified by STRING, 11 proteins were involved in protein-protein interactions network and two proteins with no interaction). Co-Expressed proteins: GC-CRP, SAA1-CRP, LBP-SAA1, C3-MBL2, C3-SAA1, C3-LBP, CRP-MBL2, GC-MBL2, LBP-MBL2, CFHR5-MBL2, LBP-CFHR5 and CRP-CFHR5.

molecular function of most proteins and "cellular process" (22.9%) was the major biological process. Identified proteins mainly originated from "cell" part (39.2%).

STRING analysis showed no protein-protein interaction between 10 candidate biomarkers. In addition, pathway analysis according to KEGG database revealed that candidate biomarkers were involved in "proximal tubule bicarbonate reclamation", "bile secretion" and "hematopoietic cell linage".

DISCUSSION

Here, we evaluated serum and urine proteome of patient with biopsy proven LN at the time of biopsy and followed them for four years. We suggested a panel of plasma and urine biomarkers that may help predicting response to treatment at diagnosis. Seventy-nine discriminating proteins in serum samples and 278 proteins in urine samples were identified, of which 20 and 10 with highest fold change were selected as biomarkers, respectively. By applying unsupervised PCA and supervised OPLS-DA models, we were able to discriminate patients achieving complete remission from those with partial remission in serum samples, while in urine samples, only OPLS-DA scores plot of samples discriminates patients with partial and complete response. Pathway analysis revealed complement pathway in serum samples. Regarding Biomarkers with higher fold change and AUC did not show any protein-protein interaction, and pathway analysis pointed out proximal tubule bicarbonate reclamation pathways, which might be due to unspecific tubular damage. These biomarkers are involved in different aspects of pathogenesis and activity of LN.

Heighten apoptosis along with impaired clearance

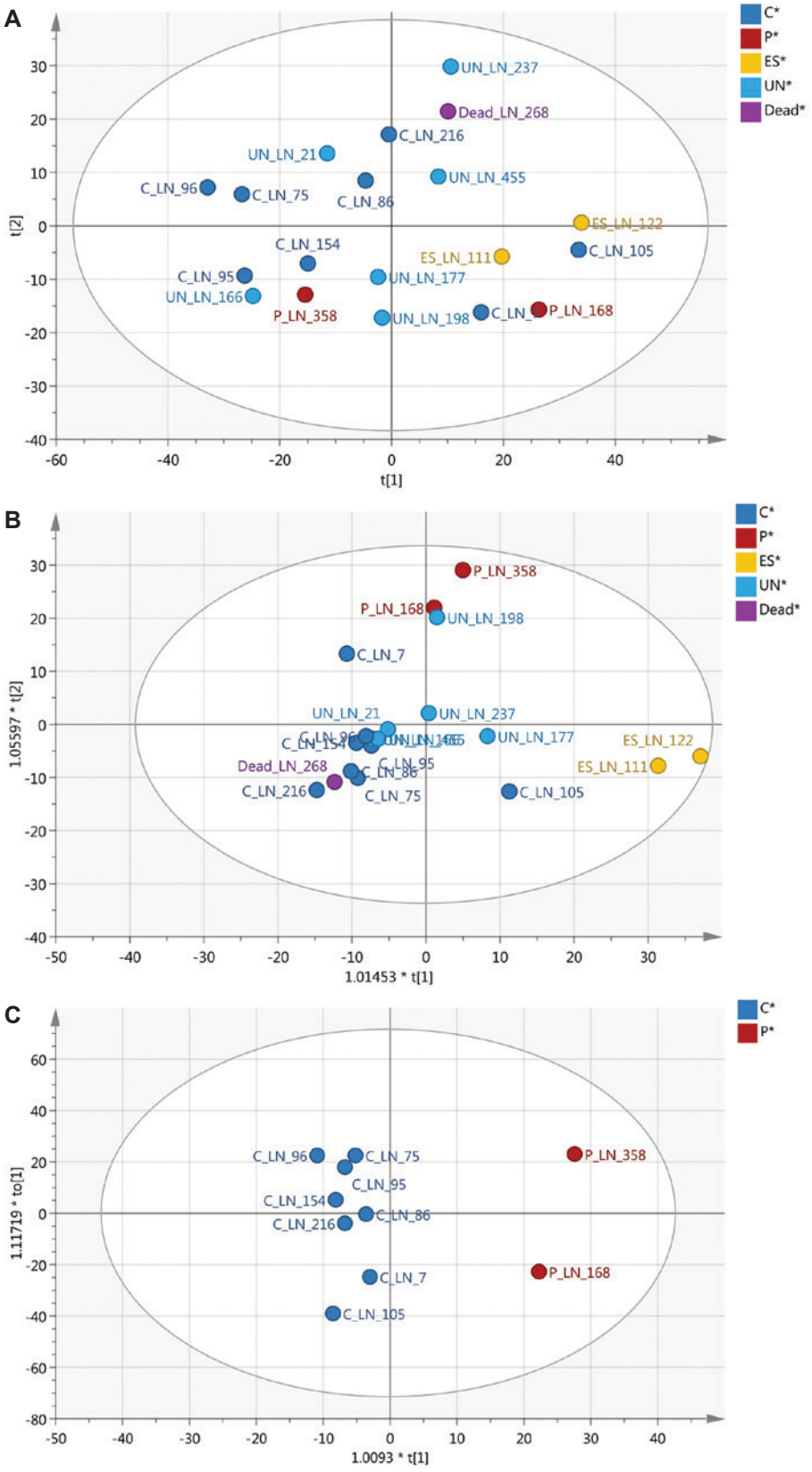
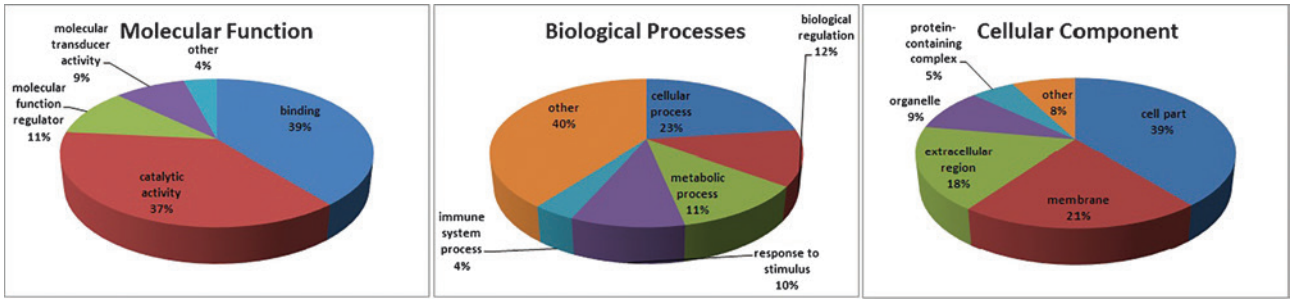


Figure 5. A) Unsupervised PCA Scores Plot Based on 1381 Quantified Proteins Across All 19 Urine Samples, B) OPLS-DA Scores Plot Based on 1381 Quantified Proteins Across All 19 Urine Samples, C) OPLS-DA Score Plot for Two Groups of Patients by Urine Samples (Abbreviations: LN, lupus nephritis; C, completed remission; P, partial remission; UN, unknown; ES, end-stage.

Table 3. Differentially Expressed Urine Proteins in Lupus Nephritis Patients with Complete Remission Compared to Partial Remission

Protein Names	Gene Names	Fold Change*	Change Direction	AUC**
Low Affinity Immunoglobulin Epsilon Fc Receptor	FCER2	45.32	Up	1
Epidermal Growth Factor Receptor Kinase Substrate 8-like Protein 2	EPS8L2	33.04	Up	1
Src Substrate Cortactin	CTTN	20.34	Up	0.93
Flavin Reductase (NADPH)	BLVRB	11.84	Down	0.93
Aquaporin-1	AQP1	7.05	Down	0.92
UPF0669 Protein C6orf120	C6orf120	25.59	Down	0.9
Platelet Glycoprotein V	GP5	7.88	Down	0.9
Sodium/potassium-transporting ATPase Subunit Beta-1	ATP1B1	14.13	Up	0.85
Ubiquitin-like Modifier-activating Enzyme 1	UBA1	23.88	Up	0.83
Protocadherin Alpha-8	PCDHA8	8.32	Down	0.83

*Fold Change Based on Peak Intensity
**AUC: area under curve



Supplementary File. Gene Ontology Enrichment Analysis of 278 Most Important Proteins in Urine

of apoptotic bodies is one of the main proposed aspects in pathogenesis of SLE, which can lead to exposure of intracellular antigens to immune system and autoantibody formation.[13] Enhanced oxidative stress and H_2O_2 within lymphocyte makes them susceptible to induced apoptosis and therefore autoantibody formation.[14] On the other hand, H_2O_2 production is enhanced by a prooxidant enzyme named prenylcyteine oxidase, which cleaves prenylcysteines to cysteine and produces a molecule of H_2O_2.[15] Antioxidant defense system includes catalase, superoxide dismutase, and glutathione peroxidase. Studies have demonstrated reduced activity of glutathione peroxidase and antibody against catalase in SLE.[16,17] Flavin reductase is a key enzyme of respiratory chain and supply the source of cell hydrogen for many oxidoreduction reactions including glutathione reduction which participates in antioxidant defense system.[18] Our data showed higher serum glutathione peroxidase and catalase, lower prenylcyteine oxidase levels and reduced urinary flavin reductase among patients with complete remission, which might be a sign of heightened defense against oxidative stress. Thus, oxidative stress could theoretically lessen the chance of therapeutic response.

Furthermore, autoantibody formation in SLE promotes inflammation, and markers of acute inflammation are correlated with disease activity. Along with well-known acute phase reactant, C-reactive protein (CRP), a novel inflammatory marker, serum amyloid A (SAA), is found in patients with active SLE in comparison with patients with inactive disease. SAA level was correlated with CRP and disease activity scores.[19] Another theory in pathogenesis of SLE is systemic exposure to intestinal microbiota and their lipopolysaccharides (LPS). Ayyappan *et al*, demonstrated that LPS binding to LPS binding protein (LBP) leads to macrophage activation and increased sCD14, lysozyme and CXCL16 level as markers of circulating antimicrobial response factors (ARFs), and these markers were associated with disease activity.[20] In line with the mentioned theory, serum level of LPS and acetate and tissue expression of LBP were significantly higher among patients with LN than patients with other glomerular diseases and healthy controls.[21] In rheumatoid arthritis (RA), LBP is shown to be a sensitive marker of disease activity,[22] and decrease

in its glycosylation occurs early in the course of disease is reversed by response to treatment.[23] Haptoglobin, another acute phase reactant, has been shown to be elevated in urine samples of patient with active LN [24- 26)] in several studies including ours (data not shown), and its under-expression in serum samples of responder group might be due to the increased urinary excretion. A study demonstrated that urinary haptoglobin declined following treatment.[26] Thus, along with SAA and CRP, the heightened LBP level and haptoglobin among the responder in our study may point out the more active disease and better response to immunosuppression.

Moreover, regulatory T (T reg $CD25^+/CD4^+$) cells have an important role in peripheral tolerance to self-antigens. Defects in Treg function or frequency has been proposed to be involved in the pathogenesis of SLE, as Tregs have a crucial role in control of undesirable inflammatory responses.[27] A recently portrayed marker of Tregs is peptidase inhibitor 16 (PI16). Tregs expressing PI16 along with CD25 migrate to the inflammation site in response to CCL17 and CCL20 in order to regulate the immune response.[28] In patients with active SLE, a wide range of changes in abundancy of Tregs have been reported, from reduced to even increased numbers. However, in a recent meta-analysis, the absolute number of peripheral Treg was low in patients with active SLE. Furthermore, authors showed functional modification of Tregs in active SLE.[29] Our data exhibited decreased expression of PI16 in plasma of patients with complete remission in comparison with those with partial remission. This finding can be translated to reduced number of Tregs and greater inflammatory response. The higher the inflammation, the more effective the immunosuppressive treatment.

All three complement pathways are somehow involved in pathogenesis and injury in SLE. Concerning the correlation between serum Mannose binding lectin (MBL) level and SLE activity and LN, the reports are contradictory. On one hand, patients with low MBL level were reported to be susceptible to SLE and had higher disease activity early in the course of disease. On the other hand, plasma level of MBL has been reported to directly correlated with disease activity. These findings could be explained by two theories. First theory is based on MBL function in pathogenesis of SLE. MBL acts as a double-edged sword, low level of MBL is associated with retained apoptotic bodies and antibody formation in SLE, and high levels of MBL causes complement activation and tissue damage.[30-34] The second theory is that MBL level is a function of production and consumption of complement proteins. In line with the second theory, low level of serum C3 is the consequence of complement system activation and consumption of complement system proteins.[35] The latter seems to be true in our cases. The other evidence of involvement of complement system in predicting response to treatment was lower serum complement factor H-related protein 5 (CFHR5). Along with factor H, CFHR5 works as a regulator of classic and alternative pathways. Data on the role of CFHR5 in LN is scarce. A recent study evaluated CFHR5function in LN reported that the higher serum level of CFHR5, the shorter progression free survival. In agreement with their finding, patients with lower CFHR5 show a better response to immunosuppressive therapy.[36] Overall, our findings imply that low CFHR5 in conjunction with low C3 and MBL are the results of higher complement system and disease activity, which predict better response to treatment.

Elevated Filaggrin expression in plasma proteome of patients who achieved complete response may be due to more immunologic active early disease. Autoantibodies against citrullinated filaggrin has been found in RA and SLE. These antibodies are associated with arthritis and erosive SLE,[37] and develop earlier in the course of disease.[38] Citrullination of filaggrin induced by inflammation, it seems that autoantibodies against citrullinated filaggrin leads to NET formation which is followed by activation of B cells and plasmacytoid dendritic cells, and tissue injury early in the course of SLE.[39] Elevated Filaggrin expression in plasma proteome of patients with complete response to immunosuppression in our study may be due to more immunologically active early disease.

Vitamin D binding protein (DBP) is a multifunctional protein that can be converted to macrophage activating factor (MAF) by deglycosylation. DBP-MAF can activate macrophages.[40] Reduced DBP level and the resultant decrease in MAF activity has been proposed to play a part in pathogenesis of SLE.[41] Besides, it plays a role in NET clearance, and DBP reduction

and NET accumulation can activate complement system. Furthermore, DBP can bind to leukocyte membrane and activate complement C5 system.[40,42] The etiology of decreased DBP level might be increased urinary loss. Several studies introduced DBP as a diagnostic urinary biomarker in LN including ours (data not shown).[43,44] In addition, serum DBP has been reported to be lower in patients with neuropsychiatric SLE, when compared with SLE patients and healthy control.[45] As it is evident by our results, lower plasma level of DBP among responder might be due to its role in pathogenesis of SLE and LN, yet data on the role of DBP in LN is scarce and needs further study.

EPS8L2 is a member of the Eps8 family and is a phosphorylation substrate for fibroblast growth factor receptor (FGFR) signaling pathway through its receptors and Src domains leading to actin remodeling and epithelial mesenchymal transition (EMT).[46,47] Up regulation of EPS8L2 and Src substrate cortactin in urine of responders in our study might be the indication of earlier onset of fibrotic alterations in these patients compared to non-responders.

Overall, a panel of plasma and urine biomarkers including proteins involved in oxidative stress, acute inflammation, reduction in regulatory T cells, complement pathway consumption and proximal tubule bicarbonate reclamation was suggested. Thus, we may conclude that the higher the inflammation, the most probable to response to immunosuppressive therapy.

Despite limited sample size, our data can precisely discriminate patients with possibility of partial response at diagnosis and guide physicians to make timely decisions on immunosuppressive drug adjustment.

CONCLUSION

Our suggested panel of plasma and urine biomarkers can precisely discriminate patients with possibility of complete response to treatment. It seems that the higher indices of inflammation will associate with better chance of achieving complete remission. Future studies with larger number of participants are needed to validate and bring the panel from bench to bed.

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CONFLICT OF INTERESTS

The authors declare that there is no conflict of interests regarding the publication of this paper.

REFERENCES

1. Liu G, Wang H, Le J, Lan L, Xu Y, Yang Y, Chen J, Han F. Early-stage predictors for treatment responses in patients with active lupus nephritis. Lupus. 2019; 28(3):283-289.
2. Dall'Era M, Stone D, Levesque V, Cisternas M, Wofsy D. Identification of biomarkers that predict response to treatment of lupus nephritis with mycophenolate mofetil or pulse cyclophosphamide. Arthritis Care Res (Hoboken). 2011; 63(3):351-7.
3. Liu G, Wang H, Le J, Lan L, Xu Y, Yang Y, Chen J, Han F. Early-stage predictors for treatment responses in patients with active lupus nephritis. Lupus. 2019; 28(3):283-289.
4. Wilhelmus S, Bajema IM, Bertsias GK, Boumpas DT, Gordon C, Lightstone L, Tesar V, Jayne DR. Lupus nephritis management guidelines compared. Nephrol Dial Transplant. 2016; 31(6):904-13.
5. Kalantari, S., et al., Urinary prognostic biomarkers and classification of IgA nephropathy by high resolution mass spectrometry coupled with liquid chromatography. PloS one, 2013; 8(12):p.e80830.
6. Saei, A.A., Beusch, C.M., Chernobrovkin, A. et al. ProTargetMiner as a proteome signature library of anticancer molecules for functional discovery. Nat Commun 10, 5715; 2019.
7. Cox, J. and M. Mann, MaxQuant enables high peptide identification rates, individualized ppb-range mass accuracies and proteome-wide protein quantification. Nature biotechnology, 2008; 26(12):1367-1372.
8. Tyanova, S., T. Temu, and J. Cox, The MaxQuant computational platform for mass spectrometry-based shotgun proteomics. Nature protocols. 2016; 11(12):2301.
9. Cox, J., et al., Andromeda: a peptide search engine integrated into the MaxQuant environment. Journal of proteome research, 2011; 10(4):1794-1805.
10. Wheelock, Å.M. and C.E. Wheelock, Trials and tribulations of 'omics data analysis: assessing quality of SIMCA-based multivariate models using examples from pulmonary medicine. Molecular BioSystems. 2013; 9(11):2589-2596.
11. Thomas, P.D., et al., PANTHER: a library of protein families and subfamilies indexed by function. Genome research. 2003; 13(9):2129-2141.
12. Szklarczyk, D., et al., STRING v11: protein–protein association networks with increased coverage, supporting functional discovery in genome-wide experimental datasets. Nucleic acids research. 2019; 47(D1):D607-D613.
13. Ahmadpoor P, Dalili N, Rostami M. An update on

pathogenesis of systemic lupus erythematosus. Iran J Kidney Dis. 2014; 8(3):171-84.

14. Pravda J. Systemic Lupus Erythematosus: Pathogenesis at the Functional Limit of Redox Homeostasis. Oxid Med Cell Longev. 2019; 2019:1651724.
15. Herrera-Marcos LV, Lou-Bonafonte JM, Martinez-Gracia MV, Arnal C, Navarro MA, Osada J. Prenylcysteine oxidase 1, a pro-oxidant enzyme of low density lipoproteins. Front Biosci (Landmark Ed). 2018; 23:1020-1037.
16. Shah, D., Mahajan, N., Sah, S. et al. Oxidative stress and its biomarkers in systemic lupus erythematosus. J Biomed Sci. 21, 23; 2014.
17. Bona N, Pezzarini E, Balbi B, Daniele SM, Rossi MF, Monje AL, Basiglio CL, Pelusa HF, Arriaga SMM. Oxidative stress, inflammation and disease activity biomarkers in lupus nephropathy. Lupus. 2020; 29(3):311-323.
18. Carlberg, I. and B. Mannervik, Purification and characterization of the flavoenzyme glutathione reductase from rat liver. Journal of biological chemistry. 1975; 250(14):5475-5480.
19. Wang CM, Deng JH, Mao GF, He YL, Shi X. Serum Amyloid A: A Potential Biomarker Assessing Disease Activity in Systemic Lupus Erythematosus. Med Sci Monit. 2020; 26:e923290.
20. Ayyappan P, Harms RZ, Buckner JH, Sarvetnick NE. Coordinated Induction of Antimicrobial Response Factors in Systemic Lupus Erythematosus. Front Immunol. 2019; 10:658.
21. Chan DTM, Chan PL, Zhang Q, Au KY,et al. Serum Acetate and Lipopolysaccharide Levels Correlate with Disease Activity in Patients With Lupus Nephritis. J Am Soc Nephrol. 26, 2015:596.
22. Wen W, Li Y, Cheng Y, et al. Lipopolysaccharide-binding protein is a sensitive disease activity biomarker for rheumatoid arthritis. Clin Exp Rheumatol. 2018; 36(2):233-240.
23. Ciregia, F., Baiwir, D., Cobraiville, G. et al. Glycosylation deficiency of lipopolysaccharide-binding protein and corticosteroid-binding globulin associated with activity and response to treatment for rheumatoid arthritis. J Transl Med. 18, 8; 2020.
24. Nicolaou O, Kousios A, Hadjisavvas A, Lauwerys B, Sokratous K, Kyriacou K. Biomarkers of systemic lupus erythematosus identified using mass spectrometry-based proteomics: a systematic review. J Cell Mol Med. 2017; 21(5):993-1012.
25. Anania VG, Yu K, Pingitore F, Li Q, Rose CM, Liu P, Sandoval W, Herman AE, Lill JR, Mathews WR. Discovery and Qualification of Candidate Urinary Biomarkers of Disease Activity in Lupus Nephritis. J Proteome Res. 2019; 18(3):1264-1277.
26. Aggarwal A, Gupta R, Negi VS, et al. Urinary haptoglobin, alpha-1 anti-chymotrypsin and retinol binding protein identified by proteomics as potential biomarkers for lupus nephritis. Clin Exp Immunol. 2017; 188(2):254-262.
27. Rastin M, Soltani S, Nazemian F, et al. Expression of T Helper 17 and Regulatory T Cell Cytokines and Molecules in Glomerulonephritis Class IV Systemic Lupus Erythematosus. Iran J Kidney Dis. 2016; 10(3):113-8.
28. Nicholson IC, Mavrangelos C, Bird DR, et al. PI16 is expressed by a subset of human memory Treg with enhanced migration to CCL17 and CCL20. Cell Immunol. 2012; 275(1-2):12-8.
29. Li W, Deng C, Yang H, Wang G. The Regulatory T Cell in Active Systemic Lupus Erythematosus Patients: A Systemic Review and Meta-Analysis. Front Immunol. 2019; 10:159.
30. Monticielo OA, Mucenic T, Xavier RM, Brenol JC, Chies JA. The role of mannose-binding lectin in systemic lupus erythematosus. Clin Rheumatol. 2008; 27(4):413-9.
31. Panda AK, Parida JR, Tripathy R, Pattanaik SS, Ravindran B, Das BK. Mannose binding lectin: a biomarker of systemic lupus erythematosus disease activity. Arthritis Res Ther. 2012; 14(5):R218.
32. Tanha N, Troelsen L, From Hermansen ML, et al. MBL2 gene variants coding for mannose-binding lectin deficiency are associated with increased risk of nephritis in Danish patients with systemic lupus erythematosus. Lupus. 2014; 23(11):1105-11.
33. Yuan ZC, Xu WD, Lan YY, et al. Association of MBL2 gene polymorphisms and systemic lupus erythematosus susceptibility: A meta-analysis. Int J Rheum Dis. 2020.
34. Lopez IL, Gasalla MG, Moreno JG, et al. Mannose binding lectin polymorphisms in systemic lupus erythematosus in Spain. European Journal of Inflammatiom.2016; 14(2): 78-85.
35. Troldborg A, Thiel S, Trendelenburg M, et al. The Lectin Pathway of Complement Activation in Patients with Systemic Lupus Erythematosus. J Rheumatol. 2018; 45(8):1136-1144.
36. Hu X, Liu H, Du J, et al. The clinical significance of plasma CFHR 1-5 in lupus nephropathy. Immunobiology. 2019; 224(3):339-346.
37. Navarro Quiroz E, Chavez-Estrada V, Macias-Ochoa K, et al. Epigenetic Mechanisms and Posttranslational Modifications in Systemic Lupus Erythematosus. Int J Mol Sci. 2019; 20(22):5679.
38. Deane KD, El-Gabalawy H. Pathogenesis and prevention of rheumatic disease: focus on preclinical RA and SLE. Nat Rev Rheumatol. 2014; 10(4):212-228.
39. Granger V, Peyneau M, Chollet-Martin S, de Chaisemartin L. Neutrophil Extracellular Traps in Autoimmunity and Allergy: Immune Complexes at Work. Frontiers in Immunology. 2019; 10:2824.
40. Bouillon R, Schuit F, Antonio L, Rastinejad F. Vitamin D Binding Protein: A Historic Overview. Front Endocrinol (Lausanne). 2020; 10:910.
41. Yamamoto N, Naraparaju VR, Moore M, Brent LH. Deglycosylation of serum vitamin D3-binding protein by alpha-N-acetylgalactosaminidase detected in the plasma of patients with systemic lupus erythematosus. Clin Immunol Immunopathol. 1997; 82(3):290-8.
42. Leffler J, Martin M, Gullstrand B, et al. Neutrophil extracellular traps that are not degraded in systemic lupus erythematosus activate complement exacerbating the disease. J Immunol. 2012; 188(7):3522-31.
43. Varghese SA, Powell TB, Budisavljevic MN, et al. Urine

biomarkers predict the cause of glomerular disease. J Am Soc Nephrol. 2007; 18(3):913-22.

44. Go DJ, Lee JY, Kang MJ, et al. Urinary vitamin D-binding protein, a novel biomarker for lupus nephritis, predicts the development of proteinuric flare. Lupus. 2018; 27(10):1600-1615.
45. Chen C, Geng L, Xu X, et al. Comparative proteomics analysis of plasma protein in patients with neuropsychiatric systemic lupus erythematosus. Ann Transl Med. 2020; 8(9):579.
46. Cunningham, D.L., et al., Novel binding partners and differentially regulated phosphorylation sites clarify Eps8 as a multi-functional adaptor. PloS one, 2013; 8(4):e61513.
47. Auciello G, et al., Regulation of fibroblast growth factor receptor signalling and trafficking by Src and Eps8. Journal of cell science, 2013; 126(2):613-624.

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