Urine Specific Proteins and Alpha-1 Antitrypsin Concentrations to Assess the Severity of Lupus Nephritis

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ABSTRACT

Current biomarkers for evaluating disease activity or severity in lupus nephritis (LN) are considered to be unsatisfactory. Pathological changes in glomerular basement membrane and selectivity of electrical discharge are causing specific patterns of urine proteins excretion. Together with alpha-1 antitrypsin (AAT), they are expected to become new biomarkers to assess LN activity.

Seventy-one urine samples were collected from healthy controls and LN patients. Patterns of urine specific proteins were determined using column chromatography and SDS-PAGE tests, LN activity was calculated using SLEDAI-renal domain score, and AAT concentrations was measured by ELISA.

The majority of proteins in the control group have molecular weights of >66 kDa (88%) and 21- to 25-kDa proteins were observed only in the case group. The p values for differences in urine AAT concentration between active LN and healthy controls, inactive LN and healthy controls, and active LN and inactive LN were 0.004, 0.046, and 0.054, respectively, whereas those for urine AAT/creatinine ratio were 0.489, 0.019, and 0.915, respectively.

There were differences in the patterns of the molecular weight of proteins and urine AAT concentrations between case group and control group. However, no such differences were identified between active and inactive LN.

Keywords: lupus nephritis activity, patterns of urine specific proteins, urine alpha-1 antitrypsin.

INTRODUCTION

Lupus nephritis (LN) is a serious manifestation that often occurs in systemic lupus erythematosus (SLE). The prevalence of LN is estimated to be between 31% – 65% with an average of 40% develops early in the course of SLE. It is more prevalent in Asians and Blacks than other races. Although its etiology is unknown, SLE occurrence has been linked to several predisposing factors such as genetic abnormalities, viral infections, and hormonal disorders (Longo et al, 2012; Kwangwoo et al, 2012). The prevalence of LN in Malang is quite high and often lead to kidney failure. A study conducted by Kusworini et al. (2010) reported that among the 30 SLE patients who underwent kidney biopsy, 73% exhibited severe LN (class III, IV, and v). This study also mentioned that of the 8 SLE patients without proteinuria, 37.5% showed histopathological characteristics of LN class III and IV.

Since SLE patients with nephritis are often asymptomatic, urinalysis should be performed on all patients suspected of having SLE. Current laboratory markers for LN such as proteinuria, urine protein creatinine ratio, creatinine clearance, anti-dsDNA, and complement levels are still

unsatisfactory. Renal biopsy plays an important role in LN, not only as the "gold standard" for establishing the diagnosis but also as a useful tool for assessing prognosis and monitoring therapy. However, it is an invasive and inconvenient procedure. Currently, extensive studies have been done to discover biomarkers that could replace the need for a renal biopsy in LN (Hsieh et al, 2012; Susianti et al, 2015).

Urine proteomic study to find biomarkers in LN has not been widely conducted (Santucci et al, 2013). Varghese et al. (2007) reported that in different kidney disease and LN classes, there were pathological changes in the size of the glomerular basement membrane and specific electrical charge selectivities, causing a specific pattern of excreted plasma protein in particular disease and histopathological class. A proteomic-based study conducted by Zhang et al. (2008) was managed to find several low-molecular weight proteomes (30 kDa) in the serial urine samples of LN patients as predictive biomarkers of LN flares. In Indonesia, there has been no data regarding the specific proteins in the urine of LN patients. Therefore, this study aimed to identify specific proteins in the urine of LN patients which can be developed into biomarkers for diagnosing LN and assessing the severity of the disease.

Alpha-1 antitrypsin (AAT) is a proteolytic enzyme which plays a major role in the normal physiological processes such as angiogenesis, intravascular fibrinolysis, and wound healing. This enzyme acts to prevent neutrophil elastase activity, a potent protease which is capable of breaking down elastic fibers dan other structural proteins. A study by Ganji, Jazii, & Sahebghadam-Lotfi (2012) has demonstrated a significant correlation between serum AAT and acute phase protein (CRP). Recent studies have proved that AAT not only acts as a protease inhibitor, but also as an anti-inflammatory agent, immunomodulator, and antimicrobial agent, and have been characterized as an important protein in certain diseases (Janciauskiene et al., 2011; Baraldo et al., 2015).

Active LN is established based on an assessment of SLE activity. The measurement of disease activity in SLE is important to evaluate the outcome or severity of the disease. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) is one instrument that often used to determine disease activity and has been widely accepted as a means to monitor SLE activity in clinical practice or for research (Quimby et al., 2013).

MATERIALS AND METHODS

This analytical observational study was conducted at the Laboratory of the Faculty of Mathematics and Natural Sciences, Brawijaya University, Malang, Indonesia, from March 2015 to October 2015.

Urine sample collection.

Random urine samples, which were taken at any time of the day and not on a specified period of time, were collected using the mid-stream method and stored frozen at-80^{o}C until analysis.

Column chromatography and SDS-PAGE tests.

Chromatography is a method used to separate solutes (components to be tested) in a sample, in which the solutes are distributed between a stationary phase and a mobile phase. This study used Unicorn 6.1, gel filtration superdex 122 chromatograpic assay. After peak patterns were obtained from the chromatogram, samples will be subjected to examination using SDS-PAGE electrophoresis. The procedure was as follows: samples were applied to the top of

the support medium and an electrical charge was given in a specified voltage within a certain period of time so that at the end of the process, molecules of different sizes separated into distinct bands. The end results were the separation of proteins according to their molecular weights.

Urine alpha-1 antitrypsin (AAT) concentrations and AAT/creatinine ratio measurement.

Urine AAT concentrations was measured by sandwich enzyme-linked immunosorbent assay (ELISA) using specific antibodies for human AAT according to the procedures included in the kit package (E0753Hu, Bioassay Technology Laboratory). Urine creatinine was measured using Roche Cobas c501 chemistry analyzer which employed enzymatic colorimetric method. Urine AAT/creatinine ratio was calculated by dividing urine AAT concentrations by urine creatinine concentrations.

Lupus nephritis disease activity.

Lupus nephritis disease activity was determined using the renal domain score of SLEDAI. This scoring system is consisted of four kidney-related parameters which can be obtained by urinalysis:

- **Urinary cast**: finding of granular or erythrocyte cast-score 4; no granular or erythrocyte cast – score 0;
- **Hematuria**: erythrocyte >5/hpf – score 4; erythrocyte <5/hpf without infection, kidney stones, or others – score 0;
- **Proteinuria**: proteinuria >0.5 g/24 hours or .3+ – score 4; proteinuria <0.5 g/24 hours or ≤3+ – score 0;
- **Leukosituria/pyuria**: leukocyte >5/hpf – score 4; leukocyte <5/hpf without urinary tract infection – score 0.

The total score is between 0 to 16 (0, 4, 8, 12, 16). Lupus nephritis was defined as active when the score is >0 and inactive when the score was 0 (Susianti et al., 2015).

Ethics.

This study has been approved by the Ethics Committee of the Faculty of Medicine, Brawijaya University, Malang, Indonesia.

Statistical analysis.

Data were shown as mean ± SD and differences in mean values between groups were analyzed using the Mann-Whitney test. Protein patterns were describe descriptively. P value of <0.05 was considered to be statistically significant.

RESULT AND DISCUSSION

Characteristics of the subjects in this study, which consists of 71 individuals, are shown in Table 1. These individuals were subsequently divided into 2 groups: control group, which consists of healthy controls, and case group, which was composed of SLE patients with inactive or active LN. Table 2 and Table 3 shows the number of protein peaks according to the results of column chromatography and the molecular weight of protein bands according to the results of SDS-Page test, respectively. The patterns of protein peaks for each group are displayed in Figure 1.

Table 1. Characteristics of healthy controls and SLE patients with LN.

Characteristics	Healthy controls (n = 23)	Inactive LN (n = 25)	Active LN (n = 23)
Age (range)*	19 – 45 years	18 – 46 years	18 – 37 years
Urinalysis results			
Proteinuria (n, %)			
Negative	23 (100)	16 (64)	6 (26)
1+	0	5 (2)	3 (13)
2+	0	4 (16)	9 (39.2)
3+	0	0	5 (21.8)
Hematuria (n, %)			
≤ 5/hpf	23 (100)	10 (100%)	5 (21.7)
> 5/hpf	0	0 (0 %)	18 (78.3)
Leukocyturia (n, %)			
≤ 5/hpf	23 (100)		4 (17.4)
> 5/hpf	0		19 (82.6)
Granular cast (n, %)			
Negative	23 (100)		19 (82.6)
Positif	0		4 (17.4)

*Statistical analysis showed no significant differences between control group and case group.

Table 2. The number of protein peaks in column chromatography.

Number of peaks	Healthy controls n (%)	Inactive LN n (%)	Active LN n (%)
6	-		1 (4.4)
7	-	1 (4)	-
8	5 (21.7)	3 (12)	4 (17.4)
9	8 (34.8)	4 (16)	6 (26.1)
10	8 (34.8)	8 (32)	5 (21.7)
11	2 (8.7)	3 (12)	3 (13)
12	-	1 (4)	1(4.4)
13	-	2 (8)	3(13)
14	-	2 (8)	-
15	-	1 (4)	-

Table 3. The molecular weight of protein bands according to the results of SDS-PAGE test.

Molecular weight of bands (kDa)	Healthy controls n (%)	Inactive LN n (%)	Active LN n (%)
90-86	2 (8)	5 (7)	3 (4.3)
85-81	0	3 (4,3)	3 (4.3)
80-76	0	5 (7)	1 (1.4)
75-71	0	7 (9.8)	11 (15.7)
70-66	20 (80)	13 (18.4)	10 (14.2)
65-61	0	2 (2.8)	3 (4.3)
60-56	0	9 (12.6)	11(15.7)
55-51	0	2 (2.8)	1 (1.4)
50-46	0	5(7.0)	6 (8.6)
45-41	0	3 (4.3)	5 (7.2)
40-36	1 (4)	0	0
35-31	0	0	0

Molecular weight of bands (kDa)	Healthy controls n (%)	Inactive LN n (%)	Active LN n (%)
30-26	2 (8)	6 (8.5)	7 (10)
25-21	0	10 (14)	9 (12.9)
20-16	0	0	0
15-10	0	1 (1.5)	0

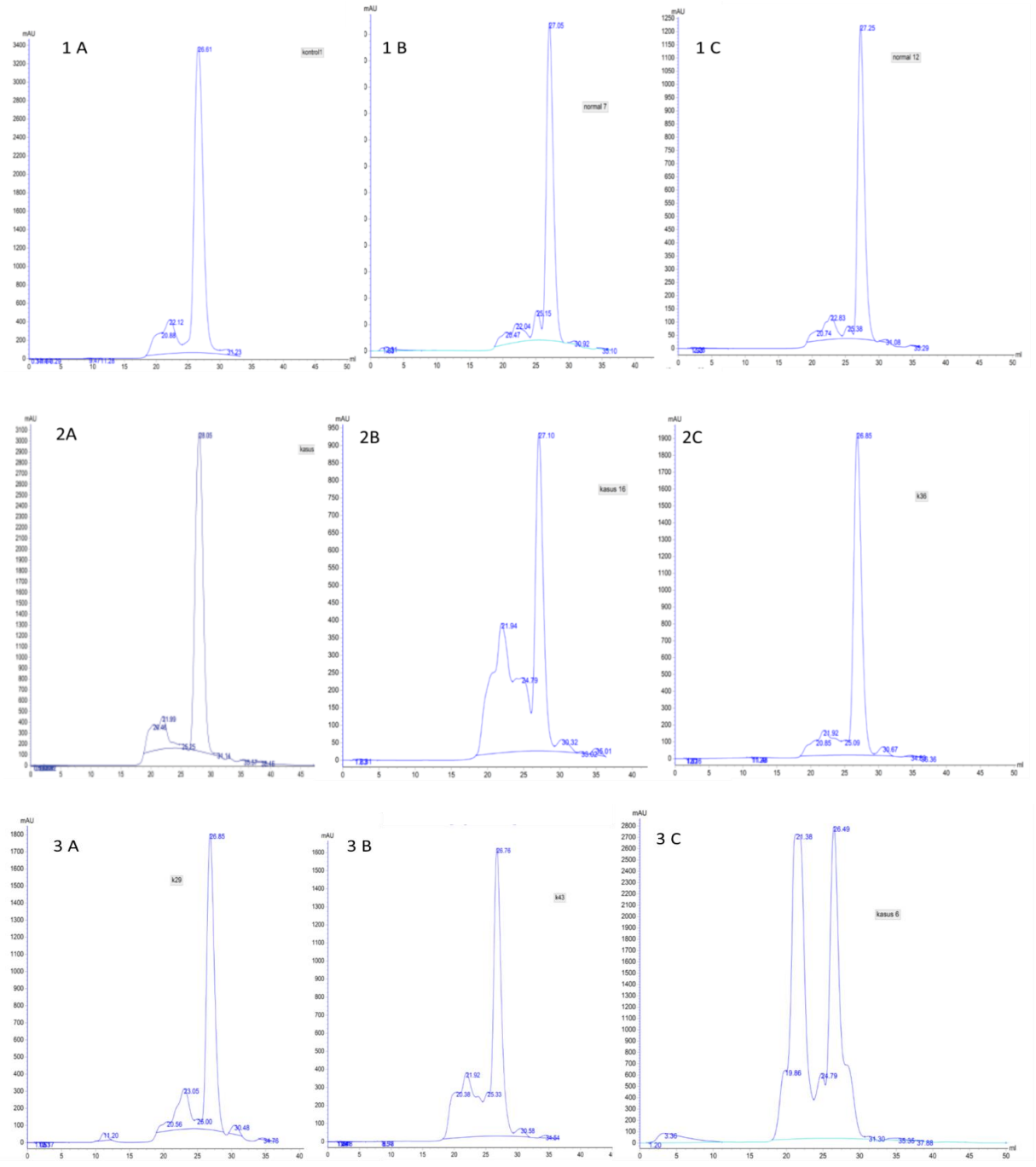

Figure 1. Chromatograms displaying the pattern of peaks in healthy controls (1A-1C), inactive LN (2A-2C), and active LN (3A-3C).

Urine AAT concentrations and urine AAT/creatinine ratio are shown in Table 4. Correlation test between the urine AAT concentrations and LN activity scores demonstrated a correlation coefficient (r) of 0.141 (p value=0.522), whereas that between the urine AAT/creatinine ratio showed a r of 0.104 (p value=0.348).

Table 4. The urine AAT concentrations and urine AAT/creatinine ratio.

Parameters	Healthy controls (mean ± SD)	Inactive LN (mean ± SD)	Active LN (mean ± SD)
Urine AAT concentrations (mg/ml)	4.84 ± 1.56	6.04 ± 2.15	6.47 ± 1.91
Urine AAT/creatinine ratio	0.11 ± 0.15	0.22 ± 0.30	0.16 ± 0.25

The average age was similar among the three groups. The average age and the age range were 28 years (19-45 years), 31 years (18-46 years), and 26 years (18-37 years) for healthy controls, inactive LN, and active LN, respectively. All of the subjects in this study were women. These findings are consistent with the previous review conducted by Almaani, Meara, & Rovin (2016) which stated that SLE is more frequently observed in women than men, with a ratio of 8:1, and often occurred during reproductive years.

The urinalysis results of this study indicated that there were glomerular damages characterized by the presence of proteinuria, hematuria, and urinary casts in active LN patients. These results agree with the findings of Saxena et al. (2008), in which proteinuria in LN patients suggests kidney damage and urinary sediment may be characteristic of increased disease activity in LN.

Patterns and types of urine specific proteins.

Column chromatographic test in this study demonstrated that the urine specific proteins of the control group did not have 12, 13, 14, and 15 peaks. This suggests that there are additional peaks that are not owned by the control group. This additional peak is possibly a type of protein that is not found in healthy controls. Urine specific proteins of active LN group did not have 14 and 15 peaks, whereas those of inactive LN group did not have 6 peaks.

Varghese et al. (2007) published a report stating that in different kidney diseases and LN classes, there were pathological changes in the glomerular basal membrane sizes and specific electrical charge selectivities. This caused specific patterns of excreted urine proteins in certain diseases and histopathological classes. Suzuki et al. (2008) reported that urine proteomic patterns in children with LN were consisted of 8 protein biomarkers They also consistently found mass spectral peaks at 2, 22, 23, 44, 56, 79, 100, and 133 in patients with LN class III, IV, and V.

In this study, the majority of protein bands in the control group had sizes of >66 kDa (88%), whereas those of the case group (active and inactive LN) had sizes of <66 kDa. This was probably due to the existence of several low molecular weight (LMW) proteins in the case group which were not found in the control group. Protein bands with sizes ranging from 21 to 25 kDa were quite commonly found in the case group, but not in the control group. These proteins are needed to be further identified. The findings of the present study are similar to the results of the study conducted by Zhang et al. (2008), who was able to identify low molecular weight proteomes (<30 kDa) in

serial urine samples of LN patients as predictive biomarkers of LN flare.

Urine alpha-1 antitrypsin concentrations and urine AAT/creatinine ratio.

Alpha-1 antitrypsin is a member of the serine protease inhibitor family. This protein inhibits the activity of neutrophil elastase, a potent protease which has the ability to break down elastic fibers and other structural proteins. The AAT concentrations in serum may vary depending on the condition of an individual, with normal values ranging from 1.5 to 3.5 g/L (or from 20 to 48 µM). The amount of research on AAT-associated renal diseases is still very small (Moreno et al., 2014; Ortiz et al., 2014; Zager et al., 2014).

The results of this study showed significant differences in urine AAT concentrations between active LN and control group and between inactive LN and control group. However, no significant difference was found between active LN group and inactive LN group. Significant difference was also observed in urine AAT/creatinine ratio between inactive LN group and control group, but not between active LN group and inactive LN group and between active LN group and control group. These results are likely to be caused by higher urine AAT/creatinine ratio in inactive LN group than active LN group. Most of the patients in active LN group have already received corticosteroid treatment, which could affect the degree of inflammation that occurred.

Sarcina et al. (2016) stated that corticosteroids and immunosupressants can reduce proteinuria in IgA nephropathy. The combination of corticosteroid and immunosuppressant was more effective in reducing proteinuria levels in patients with severe IgA nephropathy. The results of the present study demonstrated that there were differences in urine AAT concentrations between case group and control group. Therefore, AAT could possibly be used as a candidate biomarker to distinguish between patients with LN and healthy controls. Correlation tests between the SLEDAI-renal domain scores and urine AAT concentrations or urine AAT/creatinine ratio showed no significant correlation among the three parameters. These findings suggest that urine AAT may be less useful for assessing the activity of LN.

Study Limitations.

Proteins with sizes of 21 to 25 kDa which appeared to be different in case group and control group could not be identified in this study due to the limited means of research. The subjects of this study were LN patients who have already received treatment. This may affect the test results, particularly in patients with active LN.

Conclusion.

- Urine protein patterns of the case group were significantly different from those of the control group and the peak was an albumin.
- The molecular weights and peak numbers of urine specific proteins, urine AAT concentrations, and urine AAT/creatinine ratios between active and inactive LN were not significantly different.
- Neither urine AAT concentrations nor urine AAT/creatinine ratio showed a correlation with SLEDAI-renal domain scores.

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