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Serum and Urinary Biomarkers Endothelin-1, Beta-2 Microglobulin, Cystatin C, Galectin-3 and Alpha-1-acid Glycoprotein; Can they Surrogate Clinical and Histological Staging in Lupus Nephritis Patients?

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Abstract

Background: Lupus nephritis (LN) affects up to 50% of patients with Systemic lupus erythematosus (SLE) and is a major cause of morbidity. It is thus essential to identify biomarkers as indices with substantial predictive power to reduce the serious sequelae. However, criteria for disease remission have not been clearly established for these indices, except for the SLE Disease Activity Index (SLEDAI).

Objectives: To investigate the relationship of non-invasively renal protein biomarkers and established measures of renal function to histologic findings in LN, and to test whether certain combinations of the above mentioned laboratory measures are diagnostic for specific histologic features of LN and to evaluate their relations to SLEDAI and chronicity.

Methods: The study was conducted on 40 SLE female patients, recruited from renal unit of Internal Medicine department and Rheumatology and Rehabilitation department, and Neurology department, Assuit and Aswan University Hospitals, Egypt from May 2011 to January 2014, Renal biopsies were evaluated using the International Society of Nephrology/Renal Pathology Society classification (ISN/RPS), and scored for Activity Index and Chronicity Index; Clinical responders (CR) were required to have ≥50% reduction in proteinuria, normal or improved renal function, and inactive urinary sediment. Histopathological responders (HR) were required to have ≥50% improvement in Activity Index In addition, 40 age and sex matched healthy persons as a control group were enrolled in the study. The GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation. Novel serum biomarkers; Endothelin-1 (ET-1), cystatin C, beta-2 microglobulin (B2M), galectin-3 (Gal-3) and alpha-1-acid glycoprotein (AAG) were collected. Urine samples from patients were collected within 2 months of kidney biopsy and assayed for the urinary biomarkers Endothelin-1 (ET-1), α1-acid glycoprotein (AAG), Cystatin C (CysC) and beta-2 microglobulin (B2M). Renal disease activity was estimated using the British Isles Lupus Assessment Group (BILAG) index.

Results: The urinary and plasma biomarkers; ET-1, AAG, Cys C and B2M and GAL-3 were statistically significantly higher in patients with LN compared to controls that is reflective of specific histologic features seen in active LN. The combination of ET-1, AAG and CRP levels plus protein: creatinine ratio was excellent in predicting LN activity. The urinary B2M together with ET-1 and AAG plus creatinine clearance was an excellent diagnostic test for LN chronicity. However, plasma and urinary Cys C showed insignificant correlation with chronicity indices with lowest sensitivity and specificity. The statistically significantly high levels of serum and urinary ET-1 and AAG were related to specific histologic findings in LN with significant positive correlations with SLEDAI and chronicity indices in renal biopsy and highest sensitivity and specificity. Notably, these plasma biomarkers were increased linearly as renal function declined whereas urinary ET-1 and AAG rose exponentially. Thus, urinary ET-1 and AAG may be considered as a useful measure of renal inflammatory disease activity while measured renal function is still normal. Nevertheless, urinary and serum B2M exhibit a statistically insignificantly positive correlations and serum GAL-3 show insignificantly statistically negative correlations with SLEDAI and chronicity indices with lowest specificity and sensitivity reflecting the difficulty of being these biomarkers were useful markers for assessing activity and detection of early disease flares in patients with LN. Conventional clinical parameters such as creatinine clearance, proteinuria, urine sediments, anti-dsDNA, and complement levels are not sensitive or specific enough for detecting ongoing disease activity in the LNs and early relapse of nephritis.

Conclusions: In this study biomarkers namely; Endothelin -1(ET-1) and α_1-acid glycoprotein (AAG) found to be associated with specific tissue changes observed in conjunction with LN activity and chronicity. The preliminary results suggest that these biomarkers may be part of a panel that in combination may eventually be able to predict histology without the need of an invasive biopsy. Currently, however we try to discover if these promising biomarkers actually alter patient outcomes and improve the lives of the patients with this life-threatening disease complication of SLE.

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Keywords: Lupus nephritis; SLE; SLEDAI; BILAG index; Renal biopsy; Lupus nephritis; Serum biomarkers; Urinary biomarkers

Introduction

Systemic lupus erythematosus (SLE) is a multisystem inflammatory autoimmune disease in which renal involvement is one of the main determinants of poor prognosis. Lupus nephritis (LN) is a common and serious complication in SLE and is associated with significant mortality and morbidity Histologic features seen on kidney biopsy constitute the current criterion standard for the diagnosis of lupus nephritis (LN) and are used to guide LN treatment. Kidney biopsy enables direct assessment of the presence and severity of acute changes due to active LN and provides insight into the chronicity of LN [1]. The general consensus is that 60% of lupus patients will develop clinically relevant nephritis at some time in the course of their illness. Prompt recognition and treatment of renal disease is important, as early response to therapy is correlated with better outcome [2]. The Pathogenesis of LN is a complex process. The pathogenic events leading to LN can be parsed into two phases: systemic events in the immune system that orchestrate autoimmunity in SLE, and local events the cellular and molecular mechanisms that drive LN pathogenesis within the kidneys in the end organs. The multiple mechanisms lead to LN may involve deposition of auto antibodies in the glomerulus, activation of complement and macrophages, cell proliferation, production of extracellular matrix proteins, pro-inflammatory cytokines and chemokines which are then linked through multiple mechanisms to cause tubular damage, tubulointerstitial inflammation and fibrosis [3,4]. Systemic lupus erythematosus (SLE) is characterized by multiple organ involvement, by production of a wide range of antinuclear antibodies and by the presence of immune complexes in the inflamed organs. Impaired clearance of cellular debris by the reticuloendothelial system is considered a key event in the initiation and maintenance of SLE. Autoantigens escaping physiological clearance may thus become excessively presented to the adaptive immune system, resulting in loss of peripheral tolerance and occurrence of a multitude of autoantibodies - the waste disposal theory. Antibodies against dsDNA are frequently found both in serum and inflammatory lesions in glomerulonephritis. The circulating levels of anti-dsDNA often correlate with disease activity, and these autoantibodies are presumed to be of pathogenetic importance in lupus nephritis**.** The glomerulus is the commonest site of kidney involvement by lupus, however, the renal interstitium and tubules, as well as the vessels may also be affected [5]. Additionally, thrombotic and inflammatory vascular lesions can affect intra-renal or systemic haemodynamics and thus contribute to disease severity [6]. So far, Glomerulonephritis is one of the commonest and most serious manifestations of systemic lupus erythematosus (SLE) [4,7]. The disease has, in general, a variable course with periods of remission and flares eventually leading to different degrees of organ system damage and to a diminished survival**.** Although significant advances in understanding its etiopathogenesis have been made over the last several years, the identification of patients with lupus depends on the clinicians' acumen and/or established criteria, according the American Rheumatism Association) in 1997. The presentation of renal disease in SLE is variable, ranging from no symptoms (detected by routine renal biopsy or "silent" lupus nephritis), trace proteinuria or urinary sediments, microscopic hematuria, pyuria or cellular casts to frank nephrotic syndrome, chronic renal insufficiency and nephritic syndrome with rapid progression leading to renal failure. Occasionally, patients may present with chronic renal failure, isolated renal insufficiency and hypertension [8]. The prognosis of lupus nephritis depends on a large number of demographic, racial, genetic, histopathological, immunological and time-dependent factors [9]. Unfavorable prognostic factors for lupus neprhitis include younger age, male sex, histological cellular crescents, fibrinoid necrosis, subendothelial deposits, glomerular scarring, tubular atrophy and interstitial fibrosis, impaired renal function at presentation, persistent hypertension, hypocomplementemia, low hematocrit, as well as delay in treatment due to problems of access to health care and poor compliance [9]. The hallmark of lupus glomerulonephritis is proteinuria and, at the present time, it is the principal urinary biomarker that is measured when screening for the disease. Timely diagnosis of lupus nephritis is still a challenge. However, the gold standard for diagnosis is renal biopsy. Renal biopsy should be considered in SLE patients with new onset of proteinuria of more than 1 g/day with and without active urinary sediments, especially in the presence of active lupus serology or impaired renal function. Some experts recommend renal biopsy at a lower threshold of proteinuria (eg. 500 mg/day). A repeat renal biopsy should be considered in patients with persistently active serological markers [10]. Because of the extremely diverse histopathology of LN, several classifications have been proposed over the past four decades– the earliest schemes being proposed by the World Health Organization (WHO) in 1974, further refined by Austin et al. [11,12]. The features of activity and chronicity in the kidney biopsy specimen categorized as a biopsy activity index (BAI) score (range 0–24) and a biopsy chronicity index (BCI) score (range 0–12) can be calculated, with higher scores representing higher LN activity or chronicity, respectively [12]. In order to further standardize definitions and to facilitate uniformity in reporting, as well as to eliminate ambiguities and inconsistencies in the WHO classification, the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification was formulated in 2003, as detailed in Tables 1 and 2. This classification defines more precisely all glomerulonephritis (GN) classes and clearly delineates activity and chronicity. Two recent studies demonstrate the superior reproducibility of the ISN/RPS classification compared with the WHO classification of LN. In a large study involving 20 centers in the UK, renal pathologists classified cases of LN using the WHO system and then reclassified the same cases using the ISN/RPS 2003 classification scheme one year later. A significantly higher interobserver reproducibility was observed using the ISN/RPS (2003) classification than using the modified WHO (1982) classification. Nevertheless, renal biopsy as an invasive modality, it can be associated with significant morbidity, as well as inadequacies due to the 'blind'-nature of the procedure. Furthermore, a one-time diagnosis is often not sufficient, as the histopathology can change over time and therapy needs to be tailored appropriately [13]. Therefore, serial

Class I Minimal mesangial lupus nephritis
Class II Mesangial proliferative lupus nephritis
Class III Focal lupus nephritis (<50% glomeruli) III(A) Active lesions III(A/C) Active and chronic lesions III(C) Chronic lesions
Class IV Diff use lupus nephritis (>50% glomeruli) Diff use segmental (IV-S) or global (IV-G) IV(A) Active lesions IV(A/C) Active and chronic lesions IV(C) Chronic lesions
Class V Membranous lupus nephritis
Class VI Advanced sclerosing lupus nephritis (≥90% globally sclerosed glomeruli without residual activity)

Table 1: International society of nephrology/renal pathology society classification of lupus nephritis (2003).

Item (Total =40)	Descriptive
1. Age "years" mean ± SD	24.80 ± 5.77
range	19.0–38.0
2- Age of onset "years" mean ± SD	22.50 ± 4.57
range	17–33.0
3- Duration of diseases "years" mean ± SD	3.01 ± 2.9
range	0.5–6
4- Education	
educated	16 (40.0%)
non educated	24 (60.0%)
5– Occupation	
yes	0%
no	40 (100%)
6- Marital status	
Single	30 (75.0%)
Married	10 (25.0%)
7-Positive Family history	
yes	13 (32.5%)
no	27 (67.5%)
8- Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)	24.10 ± 16.86
mild	9 (22.5%)
moderate	6 (15.0%)
severe	3 (7.5%)
Very severe	22 (55.0%)

Table 2: The baseline demographic, socioeconomic characteristics of studied female lupus nephritis (LN) patients.

biopsies are impractical in the monitoring of lupus nephritis. Furthermore, current markers such as proteinuria have proved to be lacking, urine protein-to creatinine ratio, creatinine clearance, anti-dsDNA and complement levels are unsatisfactory, lacking sensitivity and specificity for differentiating renal activity and damage in lupus nephritis [14,15]. Significant kidney damage can occur before renal function is impaired and first detection by laboratory parameters. Persistent proteinuria may not necessarily indicate ongoing inflammation in the kidneys; and may be contributed by pre-existing chronic lesions or recent damage in the kidneys during the course of the disease. Flares of nephritis can occur without any observable or recent increase in the degree of proteinuria [10]. Thus, novel biomarkers, non-invasive markers that are able to discriminate lupus renal activity and its severity, predict renal flares, monitor treatment response and disease progress, and stratify prognosis are necessary [10,13]. A biomarker refers to a biologic, biochemical or molecular event that can be assayed qualitatively and quantitatively by laboratory techniques. An ideal biomarker for lupus nephritis should possess the following properties: (1) Good correlation with renal activity as reflected by the degree of proteinuria and urine sediments; (2) Sensitive to change so that it can be used for serial monitoring of disease activity in the kidneys and defining treatment response and clinical remission; (3) Ability to predict renal activity/flares before an obvious change in conventional clinical parameters occurs so that early treatment/preventive strategies can be considered; (4) Specific to nephritis among patients with SLE; and (5) Specific to SLE for aiding early diagnosis of lupus nephritis. (6) In addition, a useful biomarker should be easy to assay, simple to interpret and readily available in most laboratories with a reasonable cost. Urine biomarkers appear to be more encouraging than serum biomarkers possibly because they are the direct products or consequences of kidney inflammation or injury [7,10,16]. Endothelins are 21-amino acid vasoconstricting peptides produced primarily in the endothelium having a key role in vascular homeostasis [17]. Endothelins (ETs) have 3 isoforms of this peptide, ET-1, ET-2, ET-3, have been isolated. Their biological activities cover a wide spectrum which includes regulation of hormones and neurotransmitter, cellular growth and proliferation, bronchoconstriction, natriuresis and water diuresis [18]. Endothelin-1 (ET-1) is a most potent endogenous vasoconstrictor and its concentrations in plasma are increased markedly in a number of pathologies, such as ischemia induced damage and reperfusion, vacuities of various types, congestive heart failure, systemic inflammatory response seen in septic shock syndrome and fibrosis. [19]. Although plasma ET-1 levels are not a reliable measure of vascular ET-1 production, owing to its predominantly abluminal release, urinary ET-1 excretion is independent of plasma ET-1 concentrations and is well -correlated with renal ET-1 production [20]. Alpha-1-acid glycoprotein (AAG) is an acute phase protein modulated by two polymorphic genes. It is synthesized primarily in hepatocytes and has a normal plasma concentration between 0.6-1.2 mg/mL (1-3% plasma protein). Plasma levels are affected by pregnancy, certain drugs, and certain diseases, particularly HIV [21]. Cystatin C is a low moleclar weight (13.4 kDa) protein that functions as an inhibitor of various cysteine proteases in the blood stream [22]. It is produced by all nucleated cells at a constant rate, is filtered at the glomerulus and is taken up and degraded by the proximal tubular cells of the kidney [23]. Cystatin C is known in clinical practice as a well-described serum marker of renal failure that is not dependent on age, sex or lean muscle mass. Cystatin C is becoming acknowledged as a marker of elevated risk of death from cardiovascular complications – myocardial infarction and stroke [24]. Galectin-3 is encoded by a single gene located on chromosome 14. This protein has been shown to be involved in the following biological processes: cell adhesion, cell activation, chemo-attraction, cell growth, cell differentiation, cell cycle and apoptosis [25]. Galectin-3 has been demonstrated to be involved in cancer, inflammation and fibrosis [26]. Beta-2 microglobulin (B2M) is a serum protein found in association with the major histocompatibility complex (MHC) class I heavy chain on the surface of nearly all nucleated cells [27]. Lymphocytes and tumor cells synthesize large amounts of B2M *in vitro* and are thus presumably major biosynthetic sites [28]. In the absence of MHC class I, CD8 T cells cannot develop. Low levels of B2M can indicate non-progression of HIV. Levels of B2M can be elevated in multiple myeloma and lymphoma, though in these cases amyloidosis is more common. The normal value of B2M is <2 mg/L [29].

The assessment of remission of SLE based on Global disease activity indices: Physician global assessment is generally regarded as the gold standard for disease activity. However, this assessment is subject to substantial inter-rater variability. This variation may lead to difficulties in comparing global activity in clinical research concerning patient status and the efficacy of drugs. In recent years, many disease activity indices to measure reversible inflammation in SLE have been developed and validated These include: the British Isles Lupus Assessment Group Index (BILAG) and the Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index score is a measure for chronic damage [30], the European Consensus Lupus Activity Measurements (ECLAM), Systemic Lupus Activity Measure (SLAM), and Systemic Lupus Erythematosus Disease Activity Index (SLEDAI), which provide an overall measure of activity. Index (SLEDAI), and revised versions as SLEDAI-2K and Safety of Estrogen in Lupus Erythematosus National Assessment (SELENA) SLEDAI. Each of these indices was designed primarily for longitudinal observational studies rather than for clinical trials, but the indices have been used in both types of clinical research [31]. Activity categories have been defined on the basis of SLEDAI scores: no activity (SLEDAI=0), mild activity (SLEDAI=1-5), moderate activity (SLEDAI=6-10), high activity (SLEDAI=11-19), and very high activity (SLEDAI (≥20). A flare of SLE has been defined as an increase in SLEDAI>3, and a SLEDAI score>5 is associated with a probability

of initiating or changing therapy in more than 50% of instances [32]. While definition of flares or responses to therapy based on disease activity indices have been proposed, definitions of disease remission have not been clearly established for these indices, with the exception of the SLEDAI [31]. In this study we aimed to investigate the relationship of non-invasively renal protein biomarkers and established measures of renal function to histologic findings in lupus nephritis (LN), and to test whether certain combinations of the above mentioned laboratory measures are diagnostic for specific histologic features of LN with high specificity and sensitivity and to explore their relations to systemic lupus erythematosus disease activity index (SLEDAI) and chronicity.

Patients and Methods

Subjects

This case –control observational prospective study was conducted on 40 newly diagnosed SLE female patients, their ages ranged 19-38 years old (with mean age 24.80 ± 5.77 years), recruited from renal unit of Internal Medicine, Rheumatology and Rehabilitation and Neurology departments at Assuit and Aswan University Hospitals, Egypt from May 2011 to January 2014, with varying histologic features of lupus nephritis; Renal biopsies were evaluated using the International Society of Nephrology/Renal Pathology Society classification (ISN/RPS) as shown in Table 1, and scored for Activity Index and Chronicity Index; Clinical responders (CR) were required to have ≥50% reduction in proteinuria, normal or improved renal function, and inactive urinary sediment. Histopathological responders (HR) were required to have ≥50% improvement in Activity Index. Male gender, ≥40 years old or patients have other systemic illnesses including; diabetes mellitus, chronic cardiac dysfunction such as cardiac arrhythmias, rheumatic heart diseases, cardiomyopathic or ischemic heart disease or bleeding tendency, and other Connective tissue diseases were excluded. In addition, 40 age and sex matched healthy persons as a control group were enrolled in the study. All are subjected to thorough history taking, full clinical examination with calculation of SLEDAI, peripheral hemogram, liver function tests, kidney function tests, lipogram, prothrombin time and concentration, complete urine analysis, 24 hr urinary protein, erythrocyte sedimentation rate (ESR), C-reactive protein (CRP), rheumatoid factor (RF), anti-double stranded DNA (anti-ds DNA), antinuclear antibodies (ANA), Complement 3(C3) and Complement 4(C4). The GFR was calculated using the Chronic Kidney Disease Epidemiology Collaboration (CKD-EPI) equation [33]. Novel serum biomarkers; Endothelin-1 (ET-1), cystatin C, beta-2 microglobulin (B2M), galectin-3 (Gal-3) and alpha-1-acid glycoprotein (AAG) were collected. Urine samples from patients were collected within 2 months of kidney biopsy and assayed for the urinary biomarkers Endothelin -1(ET-1), α_1-acid glycoprotein (AAG), cystatin C (Cys C) and beta-2 microglobulin (B2M). Using nonparametric analyses, levels of urinary biomarkers and established markers of renal function were compared with histologic features seen in LN, i.e., mesangial expansion, capillary proliferation, crescent formation, necrosis, wire loops, fibrosis, tubular atrophy, and epimembranous deposits. Chest X-ray, electrocardiography and echocardiography using Agilent HP SONOS 4500 PHILIPS, U.S.A. with a 3.8 MHz transducer were done for patients. Renal disease activity was estimated using the British Isles Lupus Assessment Group (BILAG) index. Diagnosis of SLE was established according to the Systemic Lupus International Collaborating Clinics (SLICC) and American College of Rheumatology (ACR) SLE classification criteria (2012); a patient is classified as having SLE if the patient has biopsy-proven lupus nephritis with ANA or anti-dsDNA antibodies or if the patient satisfies 4 of the diagnostic criteria, including at least 1 clinical and 1 immunologic criterion [34]. Lupus nephritis is defined as clinical and laboratory manifestations that meet SLE diagnostic criteria with persistent proteinuria>0.5 g per day or greater than 3+by dipstick, and/or cellular casts including red cell, hemoglobin, granular, tubular or mixed cast [35]. A review of the ACR criteria has recommended that a spot urine creatinine/protein ratio>0.5 can be substituted for the 24 hour protein measurement, and "active urinary sediment" (>5 RBC/ high-power field (hpf)>5 WBC/ hpf in the absence of infection, or cellular casts limited to RBC or WBC casts) can be substituted for cellular casts. An additional, perhaps optimal criterion is a renal biopsy demonstrating immune complex-mediated glomerulonephritis compatible with lupus nephritis [36].

Methods

Eight ml of blood were drawn from each patient and control group after an overnight fast of 12 hours. 2 ml of blood was taken on K3EDTA vaccutainer for complete blood count by Coulter Hmx USA and ESR. Four ml of blood were collected for the separation of serum for routine kidney, liver function tests and lipogram using INTEGRA 400 autoanalyzer Boehringer Mannheim-Germany. Serum also was used for measurement of C3, C4, CRP, ANA, Anti-ds-DNA. The rest of the serum was aliquated and stored refrigerated under -20°C for the estimation of B2M using ELISA from ORGENTEC Diagnostica, Germany, Cystatin C by using ELISA from Biovendor CZEH Republic Cat No. RD191009100, Endothelin-1 by using ELISA from Glory Science Co. Ltd lot no. 20120426 USA, Galactin-3 by using ELISA kit from e Bioscience lot no. 58190009 and α1 acid glycan by using ELISA from ASSAYPRO-USA cat. no. EG5001-1 according to the manufacturer"s instruction. Second morning urine sample for complete urine analysis, while the rest of the urine sample was stored under -20°C for the estimation of B2M Cystatin C, Endothelin-1 and α1 acid glycan. Two ml of blood were collected on trisodium citrate concentration 3.2% for the estimation of prothrombin time and concentration using the Sysmex CA1500 coagulometer from Siemmens.

Statistical analysis

This research is a case control study. Data collected and analyzed by computer program SPSS" ver. 21" Chicago. USA. Data expressed as mean, Standard deviation and number, percentage. Mann-whitney was used to determine significance for numeric variables. Fisher's exact test was used to determine significance for categorical variables. Spearman's rank correlation coefficient was used for correlations between groups. P-value of less than 0.05 was considered to be statistically significant.

Results

Table 3 reveals significant low complement (C3 and C4) in patient group as compared to control group. There is a significant increase in ESR, CRP between cases and controls.

Table 4 showed that the mean serum levels of Novel Biomarkers; serum and urinary ET-1(72.72 ± 30.56 and 73.52 ± 24.70 ng/L respectively), serum and urinary Cys C (2463.5 ± 1755.61 and 9.49 ± 4.25 ng/ml respectively), serum and urinary B2M (2.18 ± 3.69 and0.179 ± 0.11 µg/ml respectively), serum and urinary AAG (3212.7 ± 145.23 and 103.50 ± 12.56 ng/ml respectively) and serum Gal-3 (15.92 ± 8.60 ng/ml) were much higher in the studied LN patient group when compared to control group (3.03 ± 0.20 and 39.90 ± 2.84 ng/L for serum and urinary ET-1 respectively, 898.70 ± 195.5 and 2.01 ± 0.83 ng/mL for serum and urinary Cys C respectively, 1.68 ± 0.67 and 0.138 ± 0.06 µg/ml for serum and urinary B2M respectively, 677.0 ± 128.34 and 15.60 ± 4.98 ng/ml for serum and urinary AAG respectively and1.03 ± 0.35

Item	(n=40) patients	Controls (n=40)	P-Value
Erythrocyte sedimentation rate (ESR) mm/h (1st hour)	105.25 ± 21.48	10.25 ± 2.43	P<0.000
C-reactive protein (CRP) mg/l	28.91 ± 8.78	3.25 ± 1.43	P<0.000
Rheumatoid factor(RF) IU/ml	35.20 ± 7.35	Negative	-
Anti-double stranded DNA (anti-dsDNA) IU/ml	40.70 ± 23.04	Negative	-
Antinuclear antibodies (ANA) IU/ml	67.95 ± 29.03	Negative	-
Complement $_3$(C_3) mg/dl	49.57 ± 17.44	89.31 ± 8.4	P<0.001
Complement $_4$(C_4) mg/dl	16.40 ± 8.21	49.25±6.3	P<0.000

Table 3: Traditional biomarkers in lupus nephritis group and controls.

Novel Biomarkers	SLE Patient group (n=40)		Control group (n=40)		P-Value
	Mean ± SD	Range	Mean ± SD	Range	
Serum Endothelin-1 (ET-1) (ng/l)	72.72 ± 30.56	14.0 -112.0	3.03 ± 0.20	2.8–3.5	P<0.000
Urinary Endothelin (ET) (ng/l)	73.52 ± 24.70	34.0 - 120.0	39.90 ± 2.84	35.0–45.0	P<0.000
Serum Cystatin (Cys C), (ng/ml)	2463.5 ± 1755.61	102.0 - 7070.0	898.70 ± 195.5	200–1100	P<0.001
Urinary cystatin C (Cys C), (ng/ml)	9.49 ± 4.25	2.60 - 88.40	2.01 ± 0.83	1.1–3.0	P<0.001
Serum beta-2 microglobulin (B2M) (µg/ml)	2.18 ± 3.69	1.0-8.8	1.68 ± 0.67	0.60 – 2.5	P<0.000
Urinary beta-2 microglobulin (B2M) (µg/ml)	0.179 ± 0.11	0.10 - 0.45	0.138 ± 0.06	0.06–0.25	P=0.08
Serum alpha-1-acid glycoprotein (AAG) (ng/ml)	3212.7 ± 145.23	2800 - 3400	677.0 ± 128.34	420–800	P<0.000
Urinary alpha-1-acid glycoprotein (AAG) (ng/ml)	103.50 ± 12.56	65.00 - 115.0	15.60 ± 4.98	8.0–25.0	P<0.000
Serum galectin-3 (Gal-3) (ng/ml)	15.92 ± 8.60	0.60 - 31.20	1.03 ± 0.35	0.6–1.3	P<0.000

Table 4: Novel biomarkers levels in studied LN patient group compared to control group.

ng/ml for serum Gal-3) with high statistically significantly difference (P<0.000).

A statistically non significantly negative correlation between SLEDAI and serum Gal-3 (ng/ml) were showed (r=-0.076 with p=0.639). Notably, There were highly significant positive correlations of SLEDAI with serum and urinary ET-1 (r=0.265 with p=0.033 ng/l; r=0.742 with p=0.031 ng/L respectively); serum and urinary Cys C (r=0.238 with p=0.021 ng/l; r=0.315 with p=0.048 ng/ml respectively) and serum and urinary AAG (r=0.672 with p=0.021 ng/l; r=0.612 with p=0.048 ng/ml respectively) with non-significant positive correlations with serum and urinary B2M (r=0.107 with p=0.512; r=0.278 with p=0.316 µg/ml) as shown in Table 5.

Table 6 showed statistically highly significantly positive correlations of Activity index/24 with serum and urinary ET-1 (r=0.577 with p=0.011, r=0.860 with p=0.023 ng/l respectively) and serum and urinary AAG (r=0.807 with p=0.001, r=0.447 with p=0.011 ng/ml respectively). However, there were non-significant positive correlations of Activity index/24 with serum and urinary cystatin C (r=0.232 and r=0.4 ng/ml respectively), serum and urinary beta-2 microglobulin (r=0.145 and r=0.179 µg/ml respectively) and serum galectin-3 (r=0.383 ng/ml). Notably, it showed statistically highly significantly positive correlations of Chronicity index/12 with serum and urinary ET-1 (r=0.231 with p=0.022, r=0.742 with p=0.003 ng/l respectively) and serum and urinary AAG (r=0.721 with p=0.01, r=0.447 with p=0.011 ng/ml respectively). However, there were non-significant positive correlations of Chronicity index/12 with serum and urinary cystatin C (r=0.179 and r=0.211 ng/ml respectively), serum and urinary beta-2 microglobulin (r=0.118 and r=0.120 µg/ml respectively) and serum galectin-3 (r=0.213 ng/ml).

There were highly statistically significantly difference between the Systemic Lupus Erythematosus Disease Activity Index (SLEDAI) and the histopathological changes of Renal biopsy with increasing grade of staging; 4.72 ± 1.34, 21.90 ± 5.86, 31.76 ± 6.37 and 46.67 ± 7.23 respectively for stage I up to stage V with P<0.000 for each. Notably, There were highly statistically significantly difference between the serum ET-1 and urinary ET-1 and the histopathological changes of Renal biopsy with increasing grade of staging (47.7 ± 11.16, 53.20 ± 21.77, 64.46 ± 36.88 and 95.67 ± 36.95 for serum ET-1 (ng/l) respectively and 61.81 ± 11.70, 66.80 ± 25.95, 79.92 ± 29.87 and 97.33 ± 29.89 for urinary ET-1(ng/l) respectively) for stage I up to stage V with P<0.007 and 0.03 for each respectively. Also, there were highly statistically significantly difference between the serum AAG and urinary AAG and the histopathological changes of Renal biopsy with increasing grade of staging (3118.2 ± 70.51, 3167.3 ± 75.69, 3269 ± 87.16 and 3290 ± 88.17 for serum AAG ng/ml, respectively and 96.81 ± 2.76, 105.50 ± 5.50, 107.15 ± 8.45 and 109.6 ± 10.58 for urinary AAG ng/ml, respectively) for stage I up to stage V with P<0.02 and 0.04 for each respectively indicating active renal inflammation in patients with lupus nephritis and their usefulness in SLEDAI. However, There were highly statistically significantly difference between the serum Cys C and urinary Cys C and the increasing histopathological changes in bizarre staging pattern of renal biopsy (1874.3 ± 1300.29, 2854.3 ± 1535.61, 3287.8 ± 2088.21 and 1106.3 ± 950.4 for serum Cys C (ng/ml) respectively and 4.44 ± 2.62, 22.89 ± 11.04, 4.75 ± 2.94 and 6.70 ± 2.82 for urinary Cys C (ng/ml) respectively) for stage I up to stage V with P<0.03 and 0.04 for each respectively with highest levels in stage II. Moreover, There were statistically significantly difference between the serum B2M and the increasing histopathological changes in bizarre staging pattern of renal biopsy (2.89 ± 2.06, 4.64 ± 1.95, 4.35 ± 2.39 and 2.16 ± 0.95 µg/ml respectively with P<0.05 for stage I up to stage V and 0.04 for each respectively with highest levels in stage II. Nevertheless, There were no statistically significantly difference between increasing histopathological changes in bizarre staging pattern of renal biopsy with serum Gal-3 (14.49 ± 9.77, 14.04 ± 9.09, 18.49 ± 6.59 and 16.13 ± 10.19 ng/ml respectively and urinary B2M (0.13 ± 0.05, 0.244 ± 0.153, 0.153 ± 0.13 and 0.213 ± 0.07 µg/ml respectively highest levels in stage II) as shown in Table 7.

The serum and urinary levels of ET-1(ng/l), AAG (ng/ml) and the serum Gal-3 ng/ml showed the highest sensitivity and specificity in studied LN patients. However, The serum and urinary levels of Cys C (ng/ml) and The serum and urinary levels of B2M (µg/ml) showed the lowest sensitivity and specificity in studied LN patients as shown in Table 8.

Data collected and analyzed by computer program SPSS" ver. 21" Chicago. USA Data expressed as mean, Standard deviation and number, percentage. Mann-whitney was used to determine significant for numeric variable, also using ANOVA test. Chi. Square (Table 9) was used to determine significance for categorical variable. Person's correlation to determine significance between variables in same group.

n.s P>0.05 no significant

* P<0.05 significant

** P<0.001 moderate significance

***p<0.000 highly significance

Novel Biomarkers	SLEDAI	
	r	p-value
Serum Endothelin-1 (ET-1) (ng/l)	0.265	0.033
Urinary Endothelin (ng/l)	0.742	0.031
Serum Cystatin (ng/ml)	0.238	0.021
Urinary cystatin C (Cys C), (ng/ml)	0.315	0.048
Serum beta-2 microglobulin (B2M) (µg/ml)	0.107	0.512
Urinary beta-2 microglobulin (B2M) (µg/ml)	0.278	0.316
Serum alpha-1-acid glycoprotein (AAG) (ng/ml)	0.672	0.021
Urinary alpha-1-acid glycoprotein (AAG) (ng/ml)	0.612	0.014
Serum galectin-3 (Gal-3) (ng/ml)	-0.076	0.639

Table 5: The correlation between SLE disease activity index (SLEDAI) and the novel biomarkers in studied LN patient group.

Novel Biomarkers	Activity index/24		Chronicity index/12	
	r	p-value	r	p-value
Serum Endothelin-1 (ET-1) (ng/l)	0.577	0.011	0.231	0.022
Urinary Endothelin (ng/l)	0.860	0.023	0.422	0.004
Serum Cystatin C (Cys C), (ng/ml)	0.232	0.07	0.179	0.06
Urinary cystatin C (Cys C), (ng/ml)	0.186	0.278	0.211	0.160
Serum beta-2 microglobulin (B2M) (µg/ml)	0.145	0.373	0.118	0.461
Urinary beta-2 microglobulin (B2M) (µg/ml)	0.179	0.268	0.120	0.571
Serum alpha-1-acid glycoprotein (AAG) (ng/ml)	0.807	0.001	0.721	0.01
Urinary alpha-1-acid glycoprotein (AAG) (ng/ml)	0.447	0.011	0.742	0.003
Serum galectin-3 (Gal-3) (ng/ml)	0.220	0.172	0.213	0.187

Table 6: The correlations of the activity and chronicity indices with the novel biomarkers in studied LN patient group.

Parameters	Stage II	Stage III	Stage IV	Stage V	P-Value
Systemic Lupus Erythematosus Disease Activity Index(SLEDAI)	4.72 ± 1.34	21.90 ± 5.86	31.76 ± 6.37	46.67 ± 7.23	P<0.000
Serum Endothelin-1 (ET-1) (ng/l)	47.7 ± 11.16	53.20 ± 21.77	64.46 ± 36.88	95.67 ±36.95	P<0.007
Urinary Endothelin (ng/l)	61.81 ± 11.70	66.80 ± 25.95	79.92 ± 29.87	97.33 ± 29.89	P<0.03
Serum Cystatin C (Cys C), (ng/ml)	1874.3 ± 1300.29	2854.3 ± 1535.61	3287.8 ± 2088.21	1106.3 ± 950.4	P<0.03
Urinary cystatin C (Cys C), (ng/ml)	4.44 ± 2.62	22.89 ± 11.04	4.75 ± 2.94	6.70 ± 2.82	P<0.04
Serum beta-2 microglobulin (B2M) (µg/ml)	2.89 ± 2.06	4.64 ± 1.95	4.35 ± 2.39	2.16 ± 0.95	P<0.05
Urinary beta-2 microglobulin (B2M) (µg/ml)	0.13 ± 0.05	0.244 ± 0.153	0.153 ± 0.13	0.213 ± 0.07	P=0.127
Serum alpha-1-acid glycoprotein (AAG) (ng/ml)	3118.2 ± 70.51	3167.3 ± 75.69	3269 ± 87.16	3290 ± 88.17	P<0.02
Urinary alpha-1-acid glycoprotein (AAG) (ng/ml)	96.81 ± 2.76	105.50 ± 5.50	107.15 ± 8.45	109.6 ± 10.58	P<0.04
Serum galectin-3 (Gal-3) (ng/ml)	14.49 ± 9.77	14.04 ± 9.09	18.49 ± 6.59	16.13 ± 10.19	P=0.600

Table 7: The relationship between the novel biomarkers, systemic lupus erythematosus disease activity index (SLEDAI) with the stages of renal biopsy in studied LN patient group.

Novel Biomarkers	Sensitivity	Specificity	Cut off
Serum Endothelin-1 (ET-1) (ng/l)	100%	90%	3.43
Urinary Endothelin (ng/l)	90%	100%	45.58
Serum Cystatin C (Cys C), (ng/ml)	65.0%	100%	1289.72
Urinary cystatin C (Cys C), (ng/ml)	5.0%	90%	3.67
Serum beta-2 microglobulin (B2M) (µg/ml)	60.0%	100%	3.02
Urinary beta-2 microglobulin (B2M) (µg/ml)	22.5%	77.5%	0.258
Serum alpha-1-acid glycoprotein (AAG) (ng/ml)	90.0%	100%	933.68
Urinary alpha-1-acid glycoprotein (AAG) (ng/ml)	90.0%	100%	25.56
Serum Galectin-3 (Gal-3) (ng/ml)	90.0%	100%	1.73

Table 8: The sensitivity and specificity of novel biomarkers in studied LN patients.

	Value	df	Asymp. Sig. (2-sided)	Exact Sig. (2-sided)	Exact Sig. (1-sided)
Pearson Chi-Square	8.310[a]	1	0.004		
Continuity Correctionb	6.385	1	0.012		
Likelihood Ratio	8.696	1	0.003		
Fisher's Exact Test				0.006	0.005
Linear-by-Linear Association	8.102	1	0.004		
N of Valid Casesb	40				
a. 1 cells (25.0%) have expected count less than 5. The minimum expected count is 4.95.					
b. Computed only for a 2×2 table					

Table 9: Chi-square tests.

Discussion

Kidney involvement in patients with systemic SLE is a common and serious complication that is often associated with a poor long-term prognosis [7]. Current treatment of severe lupus nephritis is unsatisfactory in terms of both outcome and toxicity. To improve the efficacy and decrease the adverse effects of immunosuppression, it would be ideal to be able to predict the course and pathology of LN and adjust therapy appropriately. This will require biomarkers that reflect disease activity. Renal biopsy is the gold standard for providing information on the histological classes of LN and the relative degree of activity and chronicity in the glomeruli. However, it can be associated with significant morbidity, as well as inadequacies due to the 'blind'-nature of the procedure. Furthermore, a one-time diagnosis is often not sufficient, as the histopathology can change over time and therapy needs to be tailored appropriately [13]. However, it is invasive and serial biopsies are impractical in the monitoring of LN. Recently, significant effort has been put into identifying biomarkers that can anticipate impending lupus renal flare, forecast development of chronic kidney disease, or reflect kidney histology at the time of flare, monitor treatment response and disease progress, and stratify prognosis are necessary [10]. Urine is a potential source of novel biomarker discovery in LN. The advantages of urine for this purpose are its accessibility and the fact that urine components often directly reflect pathological events within the kidneys and may reflect various aspects of the renal flare cycle.

Our study showed the statistically significant higher mean levels of traditional biomarkers; ANA, Anti-ds DNA and RF with lower levels of complements C3 and C4 and higher levels of inflammatory markers; ESR and CRP in the studied LN patients. Notably, In the current study, we found highly statistically significantly differences between Glomerular filtration rate (GFR) ml/min/1.73 m^2, the traditional biomarkers; Anti-ds DNA, ANA, C3, C4 and ESR with increasing histopathological changes in renal biopsy in bizarre pattern of staging with highest levels with stage VI and V with no statistically significantly differences between increasing histopathological changes in renal biopsy in bizarre pattern of staging with CRP and RF.

Although a large number of novel biomarkers have been studied in LN, none of them have been rigorously validated in large-scale longitudinal cohorts of patients with different ethnic background. In the current study, the novel biomarkers that are able to discriminate lupus renal activity and its severity, predict renal flares, and monitor treatment response and disease progress were clearly studied. Our study showed the high statistically significant mean levels of these Novel Biomarkers in the serum and urine of the studied LN patients.

Regarding the impact of LN on serum and urinary endothelin (ET-I), α_1-acid glycoprotein (AAG) and galactin-3 levels (GAL-3), our study showed the significant increase in the ET-1, AAG and GAL-3 in SLE patients with more higher urinary ET-1 and AAG levels with the highest sensitivity and the highest specificity of their both serum and urinary levels (100%; 90% respectively for ET-1 and 90%; 100% respectively for each AAG and GAL-3). These findings coincides with [37] who stated that ET-1 is expressed at low abundance in the normal human kidney and renal manifestations of human autoimmune diseases are associated with elevated intrarenal expression of ET-1, increased systemic ET-1 concentrations and enhanced urinary ET-1 excretion. Moreover**,** who concluded that urine contains higher concentrations of ET-1 compared to those of plasma which was mainly derived from the in situ production by the kidneys.

Furthermore, this study showed that the serum and urinary endothelin levels were grading both SLEDAI and biopsy stages; where there was a significant gradual increase in both serum and urinary ET-1 levels with the increase in biopsy stage. In support to this finding, [20] concluded that ET-1 was implicated in the development and progression of chronic kidney diseases. Furthermore, there was significant positive correlation between serum and urinary ET-1 with activity and Chronicity indices in renal biopsy. Our findings were in agreement with [38-40] who stated that endothelin both within the kidney and elsewhere, has a number of major effects including cell proliferation and inflammation which explains the significant positive correlation with activity index as well as fibrosis which explains the significant positive correlation with Chronicity index. Therefore, serum and urinary ET-1 may be considered as useful markers of renal inflammation in the early stages of inflammatory renal disease in SLE before renal function is affected. This is clearly noticed as in spite of normal GFR in stage II nephropathy patients with lupus nephritis, SLEDAI, histopathological staging activity and Chronicity indices showed significant changes. Our results are in agreement with [20] who concluded that renal ET-1 production increases as renal function declines and in subjects with SLE, urinary ET-1 may be a useful measure of renal inflammatory disease activity while measured renal function is still normal. Thus, urinary ET-1 may be suitable for noninvasive monitoring of disease activity.

Regarding α_1-acid glycoprotein (AAG), in agreement with our results, [41] found that in active LN, elevated AAG was observed both in plasma and urine and urinary levels of AAG were increased 3 months prior to the clinical diagnosis of worsening LN activity. Moreover in the current study, the urinary and serum AAG were grading both SLEDAI and biopsy stages with significant positive correlation with activity and Chronicity indices. These findings were in agreement with [1] who found that AAG was markedly increased in urine of patients with mesangial proliferation and crescents consistenting with the fact that AAG is a known marker of LN activity whose urinary levels are also elevated in other inflammatory kidney diseases [1,42] together with our results, stated that AAG has been suggested as a useful biomarker for LN.

In concordance to our results, [43,44] stated that serum Gal-3 levels were elevated in patients with SLE nephritis versus healthy controls suggesting that Gal-3 might contribute to the inflammatory process in SLE. Nevertheless, Galactin-3 exhibit insignificant correlation with SLEDAI which matched with [44] who stated that serum galactin-3 levels were not correlated with the disease activity and severity indices. However, we reported that the serum galectin-3 levels were higher in the active SLE subgroups than in the inactive SLE. Notably, our results showed that that galactin-3 does not assess the biopsy stages with insignificant correlation with activity and Chronicity indices. This finding was in contrast with [43] who found that Gal-3 reflected disease activity in SLE.

Together with our results [1], reported that there was a differential increase in levels of urinary biomarkers ET-1, AAG and serum galactin-3 that formed a pattern reflective of specific histologic features seen in active LN. Moreover, these results were in agreement with [10] who stated that these novel biomarkers are necessary to enhance the diagnostic accuracy and sensitivity of lupus renal disease, prognostic stratification, monitoring of treatment response, and detection of early renal flares. Furthermore, stated that the identification of urine biomarkers has traditionally been approached by evaluating candidate proteins chosen because of a relationship to the pathogenesis of SLE nephritis.

Regarding the impact of LN on levels of cystatin, our study showed the significant increase in their levels in the serum and urine of studied SLE patients. These results coincided with [45,46] who stated that Cys C is increased in SLE and in SLE patients with a history of nephritis even after adjustment for conventional measures of renal function. However, in the current study, Serum and urinary Cys C in spite of being specific (100% and 90% respectively) they show low sensitivity (65% and 5% respectively). The Low sensitivity of cystatin C could be attributed to the presence of different factors influencing the levels of cystatin C to be taken into account such as levels of C- reactive protein, smoking, obesity, and gender, use of glucocorticoids, age, greater height and diabetes. Moreover, white blood cell count and lower serum albumin are associated with higher levels of Cys C [47,48] reports claim a dependence of Cystatin C upon thyroid function, possibly influencing the production rate of the protein. Notably, in the current study, Serum and urinary Cys C didn't assess the biopsy stages especially in stage IV and V with significant positive correlations with both activity and Chronicity indices. To our knowledge no previous research discover the relation between Cys C and renal biopsy but, [23,49] who found no evidence that multivariate serum cystatin C-based estimates of renal function are superior to multivariate serum creatinine-based estimates. In spite of the Serum and urinary cystatin exhibited a significant positive correlation with SLEDAI in our study, we could not use their levels as useful measures of lupus nephritis and they did not greatly improve the pre-existing dilemma in LN follow up. These findings were in agreement with [46] who concluded that Cys C should not supplant current methods of assessing renal dysfunction in SLE

patients. Therefore, we could not use serum and urinary Cys C as useful measures of lupus nephritis as they did not assess biopsy stages.

The impact of LN on serum and urinary beta 2-microglobulin (β2M) concentrations was also investigated in this study. Our results showed a significant increase in serum and urinary β2M levels in studied SLE patients. This finding was in concordance with [50,51] who stated the significant higher serum levels of β2M in SLE patients. In agreement to our results, reported insignificant increase in the urinary excretion of beta2-microglobulin β2M in lupus nephritis. Notably, our study cleared that the Serum β2M had high specificity (100%) with low sensitivity (60%) and urinary β2M had low specificity and low sensitivity (77.5% and 22.5% respectively) with insignificant positive correlations with SLEDAI, Activity index/24 and Chronicity index/12. These findings were matched with [52] regarding Serum β2M, who found insignificant difference between low and high SLEDAI groups. But our results were in disagreement with [52] regarding urine β2M, as they found significant difference between low and high SLEDAI groups regarding urinary β2M. This conflict in agreement to study by Silva [53] could be explained by the fact that β2M is a low-molecular-weight protein mainly released by activated lymphocytes and most our cases of SLE patients have Lymphopenia. Notably, we found that serum and urinary β2M don't assess the biopsy stages with insignificant correlation with activity and Chronicity indices. Therefore these markers together with Cys C could not be used to assess or follow up disease activity in LN.

We concluded, according to our results, the novel biomarkers: Endothelin -1(ET-1) and α_1-acid glycoproteins (AAG) are associated with specific tissue changes observed in conjunction with LN activity and chronicity with high sensitivity and specificity. Especially in combination with select established markers of renal function, the urinary biomarkers are well-suited for use in noninvasive measurement of LN activity and LN chronicity. The preliminary results suggest that these biomarkers may be part of a panel that in combination may eventually be able to predict histology without the need of an invasive biopsy. In addition they grade both SLEDAI and biopsy stages with significant positive correlation with activity and chronicity indices in renal biopsy. Especially urine samples make the data more widely applicable, noninvasive and readily available.

We are optimistic that biomarkers will emerge in future LN scoring with the ability to positively affect management and decrease the morbidity and mortality of this difficult to control disease manifestation. Currently, however, we try to discover if these promising biomarkers actually alter patient outcomes and improve the lives of the patients with this life-threatening disease complication of SLE. Therefore, further larger studies along this line of research are clearly necessary to provide further evidence to the role of these markers in the pathophysiological process of lupus and to predict the course of LN, the severity of kidney pathology and to assess treatment and its response.

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