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Interleukin (IL) 16: a candidate urinary biomarker for proliferative lupus nephritis

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ABSTRACT

Objective Lupus nephritis (LN) is a severe manifestation of systemic lupus erythematosus (SLE). The pathogenesis is incompletely understood and diagnostic biomarkers are scarce. We investigated interleukin (IL) 16 as a potential biomarker for LN in a well-characterised cohort of patients with SLE.

Methods We measured urinary (u-) and plasma (p-) levels of IL-16 in predefined patient groups using ELISA: LN (n=84), active non-renal SLE (n=63), inactive non-renal SLE (n=73) and matched population controls (n=48). The LN group included patients with recent biopsy-confirmed proliferative (PLN, n=47), mesangioproliferative (MES, n=11) and membranous (MLN, n=26) LN. Renal expression of IL-16 was investigated by immunohistochemistry. Associations between IL-16 measurements and clinical parameters and the diagnostic value for LN were explored.

Results p-IL-16 was detected in all investigated cases and high p-IL-16 levels were observed in patients with active SLE. u-IL-16 was detected (dt-u-IL-16) in 47.6% of patients with LN, while only up to 17.8% had dt-u-IL-16 in other groups. In the LN group, 68% of patients with PLN had dt-u-IL-16, while the proportions in the MLN and MES groups were lower (11.5% and 45.5%, respectively). The highest u-IL-16 levels were detected in the PLN group. In the regression model, u-IL-16 levels differentiated PLN from other LN patient subgroups (area under the curve 0.775–0.896, p<0.0001). dt-u-IL-16 had superior specificity but slightly lower sensitivity than elevated anti-double-stranded DNA and low complement C3 or C4 in diagnosing PLN. A high proportion of LN kidney infiltrating cells expressed IL-16.

Conclusions We demonstrate that detectable u-IL-16 can differentiate patients with PLN from those with less severe LN subtypes and active non-renal SLE. Our findings suggest that u-IL-16 could be used as a screening tool at suspicion of severe LN. Furthermore, the high IL-16 levels in plasma, urine and kidney tissue imply that IL-16 could be explored as a therapeutic target in SLE.

WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Lupus nephritis (LN) is a severe manifestation of systemic lupus erythematosus (SLE).
⇒ Kidney biopsy is a gold standard method for establishing an LN diagnosis.
⇒ Interleukin (IL) 16 is a novel cytokine with increasing evidence of its importance in SLE.
⇒ It is of interest if IL-16 in circulation or urine could serve as a biomarker for SLE and LN.

WHAT THIS STUDY ADDS

⇒ The study demonstrates that detection of IL-16 in urine is associated with proliferative LN (PLN), the most severe form of LN.
⇒ We could show that urinary IL-16 could discriminate PLN from other types of LN and other manifestations of active SLE.

HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE OR POLICY

⇒ Our findings indicate that urinary IL-16 is a candidate biomarker for PLN and if validated could possibly replace kidney biopsies at LN suspicion.

INTRODUCTION

Lupus nephritis (LN) is a severe manifestation of systemic lupus erythematosus (SLE).[1] Renal biopsy is the gold standard method for establishing an LN diagnosis. Biopsies yield crucial information on histopathological class and activity and are needed to guide proper treatment decisions.[2] However, a biopsy is an invasive procedure associated with potential complications. Therefore, a reliable non-invasive diagnostic method that can give similar information would significantly enhance the management of patients with LN.[3 4] Consequently, it is of importance to identify potential biomarkers that can capture and differentiate the phenotypes of patients with SLE, including LN and different LN classes.

Interleukin (IL) 16 is an immunomodulatory cytokine with reported roles in several autoimmune conditions.[5–7] We have earlier reported increased levels of circulating IL-16 in patients with SLE and noted high levels in patients with LN.[8] IL-16 was also identified as the most significantly enriched cytokine in

both LE skin lesion and LN urine proteomes.[9 10] IL-16 is a cytoplasmic protein cleaved by caspase-3 on various triggers. After cleavage, a mature C-terminal IL-16 is secreted and involved in chemoattraction, migration and activation of CD4 T cells and may also mediate effects by binding to C-C chemokine receptor type 5 (CCR5) and CD9.[11 12] The N-terminal pro-IL-16 translocates to the nucleus and may impact cell cycle.[12 13] Most types of immune cells and also various epithelial cells may express IL-16.[14–16]

In this study, we measured the IL-16 levels in plasma (p-) and urine (u-) in a large and well-characterised SLE cohort, which included patients with biopsy-proven LN and active non-renal SLE, as well as inactive SLE and population controls (pCs). We also explored if circulating or urinary IL-16 could serve as a biomarker for LN and assessed its diagnostic value. Additionally, we explored the expression of IL-16 in kidney biopsies.

PATIENTS AND METHODS

Patients

Patients were recruited from SLE and LN cohorts at Karolinska University Hospital (KS) in Stockholm, Sweden (table 1). All patients fulfilled at least four of the 1982 American College of Rheumatology[17] and/or the SLICC (Systemic Lupus International Collaborating Clinics).[18]

Table 1 Characteristics of the cohort: SLE patients with active LN, ANR-SLE, INR-SLE and population controls

	LN	ANR-SLE	INR-SLE	Population controls	P value
Patients, n	84	63	73	48	ns
Sex, female (%)	89.3	90.5	81.3	89.0	ns
Age, M (SD)	36.2 (13)	37.9 (12)	47.7 (16)	38.6 (15)	0.008
Disease duration	2 (0–8)	6 (1–12)	7 (4.5–15.5)	NA	<0.0001
Ethnicity (Caucasian/Asian/African/Hispanic), n	75/3/2/4	51/6/6/0	62/6/2/3	44/2/0/2	ns
p-creatinine (µmol/L)	68 (57–83)	66 (58–73)	67 (58–76)	63 (56–71)	ns
eGFR, mL/min/1.73^2	105 (77–123)	103 (89–121)	95 (79–109)	NA	0.09
u-ACR (mg/mmol)	45.3 (19.0–76.1)	2.8 (1.5–3.8)	1.4 (0.9–2.4)	0.4 (0.3–0.5)	<0.0001
Positive a-dsDNA*, %	64	46	25	NA	<0.0001
Low C3†, %	70	25	0	NA	<0.0001
Low C4†, %	75	45	15	NA	<0.0001
SLEDAI-2K score	12 (8–18)	6 (4–8)	1 (0–2)	NA	<0.0001
No treatment, n (%)	14 (16.6)	7 (11.1)	23 (31.5)	NA	ns
Prednisolone, n (%)	63 (75.0)	45 (71.4)	29 (39.7)	NA	<0.0001
Prednisolone (mg‡), M (SD)	15 (13)	8.5 (9.4)	3.3 (5.7)	NA	<0.0001
Prednisolone ≥10 mg, %	55.5	36.5	15.0	NA	LR25.7<0.0001
Hydroxychloroquine, n (%)	39 (46)	38 (60)	35 (48)	NA	ns
DMARD any, n (%)	38 (45)	29 (46)	18 (25)	NA	<0.0001
dt-p-IL-16, n (%)	79 (100)	60 (100)	47 (100)	38 (100)	ns
p-IL-16 (ng/mL)	0.43 (0.3–0.8)	0.36 (0.2–1.0)	0.25 (0.2–0.5)	0.2 (0.16–0.5)	0.0028
p-IL-16 (ng/mL), M (SD)	6.2 (0.8)	6.09 (0.98)	5.8 (0.75)	5.7 (0.89)	ns
dt-u-IL-16, n (%)	40 (47.6)	4 (6.3)	13 (17.8)	2 (4.3)	<0.0001
u-IL-16 (pg/mL)	3§ (3§–33.1)	n-dt¶	n-dt¶	n-dt¶	<0.0001
u-IL-16 (pg/mL), M (SD)	27.0 (42.5)	5.2 (10.4)	9.8 (34.2)	3.4 (2.4)	<0.0001

All variables are presented as median and IQR, if not indicated otherwise.
*Data on a-dsDNA status were missing in 9 patients with LN, 17 with ANR-SLE and 18 with INR-SLE.
†Data on C3 and C4 status were missing in 27 patients with LN, 3 with ANR-SLE and 9 with INR-SLE.
‡Prednisolone dose or equivalent steroid dose.
§3 is an arbitrary value for non-detectable levels for analysis by non-parametric tests.
¶Only undetectable levels found in the IQR.
ACR, morning albumin to creatinine ratio; a-dsDNA, anti-double-stranded DNA; ANR-SLE, active non-renal SLE; DMARD, disease-modifying antirheumatic drugs (detailed information is provided in online supplemental material); dt, detectable (detection limit was defined by ELISA manufacturer's recommendations as >9 pg/mL); eGFR, glomerular filtration rate; IL, interleukin; INR-SLE, inactive non-renal SLE; LN, lupus nephritis; LR, likelihood ratio; M, mean; NA, data not analysed; n-dt, non-detectable; p, plasma; SLE, systemic lupus erythematosus; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; u, urine.

The predefined patient groups included 84 patients with biopsy-verified LN, 63 with active non-renal SLE (ANR-SLE) and 73 with inactive non-renal SLE (INR-SLE) with no history of LN, as well as 48 matched pCs. Demographic and clinical information was collected from the cohort databases and electronic medical records. A cohort of 11 patients with LN and available kidney biopsies were also investigated (online supplemental table 2).

SLE disease activity was assessed using the Systemic Lupus Erythematosus Disease Activity Index 2000 (SLEDAI-2K) instrument.[19] Patients with an SLEDAI-2K score ≥4 were defined as active and those with a score <4 as inactive. None of the patients in the ANR-SLE or INR-SLE group displayed clinically evident features of past or current LN. All patients with LN were recruited at clinically indicated renal biopsy.

Informed consent was acquired prior to recruitment of each subject.

Routine chemistry parameters

At inclusion, plasma and urine samples were collected and stored at −80°C. Samples from patients with LN were acquired a day before or on the day of renal biopsy prior to the biopsy procedure. Patients' p-creatinine was measured at the Department of Clinical Chemistry at KS. Renal function (estimated glomerular filtration rate, eGFR) was calculated using the Modification of Diet in Renal Disease equation.[20] Anti-double-stranded DNA antibodies (a-dsDNA) and levels of complement factors C3 and C4 were analysed according to clinical routine at the Department of Clinical Immunology at KS, where methods have changed over the years. Therefore, data are defined as positive or negative with regard to a-dsDNA and high or low in C3 and C4.

Over the years at KS, different methods have been used to estimate the grade of proteinuria and/or albuminuria. Therefore, u-albumin and u-creatinine were analysed at the same occasion on Mindray BS-380 (Shenzhen Mindray Bio-medical Electronics, Shenzhen, China) using reagents from Abbott Laboratories (Abbott Park, Illinois, USA). The u-albumin to creatinine ratio (u-ACR) was calculated to estimate the grade of albuminuria, which is a commonly used proxy for 24-hour proteinuria in nephrology.[21] According to the conversion formula, u-ACR ≥30 mg/mmol can be regarded as equivalent to ≥0.5 g/24-hour proteinuria.[21] All measurements in urine were performed according to clinical routine at the Department of Clinical Chemistry at Uppsala University Hospital.

LN cohort and renal histopathology

In total 84 patients with LN and acquired renal biopsies were included. Indications for biopsy were clinical suspicion of a first-time LN flare (n=47), or either per-protocol rebiopsies (ie, 6–12 months after induction treatment) (n=12) or at clinical signs of a new nephritis flare (n=25). All biopsies were assessed and confirmed for LN by experienced nephropathologists at the Pathology Unit at KS and were classified according to the International Society of Nephrology/Renal Pathology Society system and graded for activity and chronicity indices.[22] There were 47 patients with proliferative LN (PLN, class III or IV), 11 with mesangioproliferative LN (MES, class II) and 26 with membranous LN (MLN, class V). Three patients had mixed pattern with PLN and MLN and were included in the PLN group, while another two had mixed histopathology of MLN and MES and were included in the MLN group. Active LN was defined as histopathological activity index of 3 or more.[22]

IL-16 measurements

Plasma and urine IL-16 levels were measured using a commercial sandwich ELISA kit from MilliporeSigma (Cat# RAB0261; St Louis, Missouri, USA) according to the manufacturer's instructions. The cut-off for detectable IL-16 was 9 pg/mL. u-IL-16 measurements were performed in all cases, while plasma samples were available in 39 pCs and 47 patients with INR-SLE, 60 with ANR-SLE and 80 with LN.

Immunohistochemistry

Eleven LN kidney biopsy specimens were available for research purposes and were stained for IL-16 by immunohistochemistry (IHC). The characteristics of this cohort are displayed in online supplemental table 2. The IHC procedure is described in detail in online supplemental methods.

Evaluation of IHC staining

The sections were visualised using Leica Reichert Polyvar II light microscope. The analysis was performed semi-quantitatively by two investigators, who assessed the mononuclear cells comprising renal interstitial infiltrates. Renal parenchymal cells were excluded by visual analysis.

The proportion of kidney infiltrating inflammatory cells stained for IL-16 was estimated in per cent in representative high-power field in each section by two investigators (AH and VO) and confirmed using the manual cell counting tool in QuPath V.0.1.2 digital pathology image analysis software.[23] The ratio of cells stained positively for IL-16 versus all counted infiltrating cells in the interstitium was calculated. The indicated values are the means of the estimated proportions (online supplemental table 2).

Statistical analysis

Statistical analyses were conducted by JMP V.16 and GraphPad Prism V.9.3.6 software. Depending on data type and distribution, Student's t-test, analysis of variance (ANOVA), Mann-Whitney U or Kruskal-Wallis test was used. Correlations were estimated by Spearman's rank correlation test. Categorical variables were analysed as proportion using Fisher's exact test. Receiver operator characteristic curve (ROC) and area under the curve (AUC) were calculated. Specificity, sensitivity, positive and negative predictive values, and OR were calculated manually. P values below 0.05 were considered significant.

RESULTS

Characteristics of the cohort

Among the four investigated groups (LN, ANR-SLE, INR-SLE and pCs), there were no significant differences in sex, ethnicity or renal function, but patients with INR-SLE were older compared with the other groups (table 1). A higher proportion of patients with LN had positive a-dsDNA, consumption of C3 or C4, elevated p-creatinine, as well as higher u-ACR and SLEDAI-2K scores in comparison with the other groups (table 1).

The following were the proportions of patients with SLE who were on steroids: 75% of LN, 71.4% of ANR-SLE and 39.7% of INR-SLE, where patients with LN had the highest doses of steroids (table 1). Of the patients, 76% were on hydroxychloroquine. Forty-five per cent of patients with LN, 46% with ANR-SLE and 25% with INR-SLE were on other disease-modifying antirheumatic drugs (table 1; detailed information in online supplemental table 1).

p-IL-16 levels were highest in patients with active SLE

p-IL-16 was detected in all investigated samples. Patients with active SLE (ANR-SLE and LN pooled together) had higher p-IL-16 in comparison with INR-SLE or pC (p<0.01), while the levels did not differ between inactive patients and controls (ns). Among all four groups, patients with LN had significantly higher p-IL-16 levels in comparison with patients with INR-SLE and pCs (p<0.002), while no significant difference was found between LN and ANR-SLE (figure 1A).

u-IL-16 levels were highest in patients with LN

u-IL-16 was detected in 47.6% of patients with LN, but only in up to 17.8% of individuals in the other studied groups (Pearson's χ^2=50.7, p<0.0001). u-IL-16 levels were higher in the LN group in comparison with all other groups (p<0.0001) (figure 1B and table 1).

p-IL-16 levels did not differ among LN classes, while u-IL-16 levels were highest in PLN

Next, we investigated the characteristics of patients with LN with different histopathological classes: PLN, MLN and MES (table 2). There were no differences in sex, age, p-creatinine and eGFR among the groups. A significantly higher proportion of patients with PLN had positive a-dsDNA and low C3 and/or C4 when compared with MLN and MES groups. The highest u-ACR levels, SLEDAI-2K score and LN activity index were also observed within the PLN group (table 2).

p-IL-16 was detected in all patients with LN and the levels did not differ among the LN subsets (table 2 and figure 1C). In contrast, detectable levels of u-IL-16 (dt-u-IL-16) were present in a larger proportion of patients with PLN (68.1%), 45.5% in patients with MES and in few MLN cases (11.5%) (p<0.0001; table 3). Patients with PLN had the highest u-IL-16 levels in comparison with patients with MLN (p<0.0001) and MES (p=0.035) (table 2 and figure 1D).

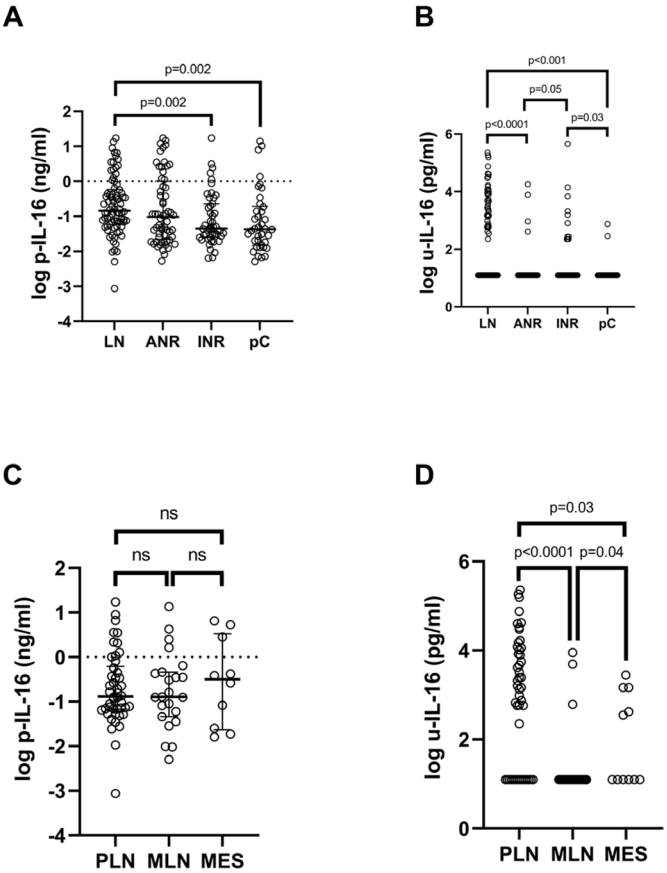

Figure 1 Distribution of plasma (p-) and urine (u-) IL-16 levels among the investigated groups. (A) Patients with LN had higher plasma levels of IL-16 in comparison with patients with INR-SLE and pCs. (B) Patients with LN had the highest urine levels of IL-16 in comparison with other investigated groups: ANR-SLE, INR-SLE and pCs. (C) p-IL-16 levels did not differ among the investigated LN classes: PLN, MLN and MES. (D) Patients with PLN had higher u-IL-16 levels than patients with MLN or MES. ANR, active non-renal; IL-16, interleukin 16; INR, inactive non-renal; LN, lupus nephritis; MES, mesangioproliferative lupus nephritis; MLN, membranous lupus nephritis; ns, not significant; pCs, population controls; PLN, proliferative lupus nephritis; SLE, systemic lupus erythematosus.

Correlations between IL-16 measurements and clinical parameters

In all patients with SLE investigated as one group, disease activity (SLEDAI-2K score) correlated moderately with u-IL-16 (r=0.36, p<0.001) and weakly with p-IL-16 (r=0.2, p=0.006). A weak correlation between p-IL-16 and u-IL-16 was seen (r=0.15, p=0.039).

In the PLN group, LN activity index correlated with u-IL-16 levels (r=0.39, p=0.007) (online supplemental figure 1A). Also, u-IL-16 correlated with p-IL-16 levels (r=0.36, p=0.014) (online supplemental figure 1B). Besides, u-ACR correlated positively with both p-IL-16 and u-IL-16 levels (r=0.35, p=0.017 and r=0.31, p=0.034, respectively) (online supplemental figure 1C; data not shown), but when patients without albuminuria were excluded correlation was no longer observed (online

Table 2 Characteristics of patients with different classes of lupus nephritis

	PLN	MES	MLN	P value
Patients, n	47	11	26	
Sex, female (%)	94	82	85	ns
Age, M (SD)	35.5 (14)	38.8 (11.8)	36.5 (13.5)	ns
Disease duration	2 (0–5)	6.5 (2–15)	0 (0–15)	ns
First biopsy, n (%)	63.8	36.4	50.0	ns
p-creatinine (µmol/L)	68 (55–82)	74 (62–83)	66 (55.5–94.5)	ns
eGFR, mL/min/1.73^2	108 (80–124)	98 (77–113)	101 (74–128)	ns
u-ACR (mg/mmol)	52.3 (38–87)	20.2 (8–42)	26.0 (14–68)	0.006
u-ACR ≥50 (mg/mmol), n (%)	27 (57.5)	2 (18.2)	9 (34.5)	ns
u-ACR ≥30 (mg/mmol), n (%)	38 (80.8)	4 (36.4)	12 (46.2)	*ns*
Positive a-dsDNA*, %	77.0	45.4	50.0	0.005
Low C3†, %	78.0	37.5	41.5	0.01
Low C4†, %	92.0	50.0	41.5	0.0004
SLEDAI-2K score	16 (12–20.5)	10 (5.5–13)	8 (4–12)	<0.0001
LN activity index	4 (3–6)	1 (1–1)	1 (0–2)	<0.0001
LN chronicity index	0 (0–1)	0 (0–2)	*0.5 (0–2.25)*	ns
No treatment, n (%)	11 (23.4)	0 (0.0)	12 (46.1)	ns
Prednisolone, n (%)	34 (72.3)	8 (72.7)	21 (80.7)	ns
Prednisolone (mg*), M (SD)	17.2 (14)	15.3 (16)	12.5 (10)	ns
Prednisolone ≥10 mg (%)	61.7	50.0	47.6	ns
Hydroxychloroquine, n (%)	21 (44.6)	5 (45.4)	13 (50.0)	ns
DMARD any, n (%)	16 (34.0)	8 (72.7)	15 (57.7)	0.04
dt-p-IL-16, n (%)	46 (100)	10 (100)	23 (100)	ns
p-IL-16 (ng/mL)	0.4 (0.3–0.8)	0.61 (0.2–1.7)	0.4 (0.26–0.7)	ns
p-IL-16 (ng/mL), M (SD)	0.69 (0.69)	0.86 (0.8)	0.67 (0.68)	ns
dt-u-IL-16, n (%)	32 (68.0)	5 (45.4)	3 (11.5)	<0.0001
u-IL-16 (pg/mL)	24 (3‡–58)	3‡ (3‡–24)	n-dt§	<0.0001
u-IL-16 (pg/mL), M (SD)	41.9 (51.4)	11.2 (10.7)	6.8 (12.0)	0.0009

All variables are presented as median and IQR, if not indicated otherwise.
u-ACR >30 corresponds to proteinuria of ≥0.5 g/24 hours).[21]
*Data on a-dsDNA status were missing in 3 of PLN and 6 of MLN.
†Data on C3 and C4 status were missing in 10 patients with PLN, 3 MES and 14 MLN.
‡3 is an arbitrary value for non-detectable levels for analysis by non-parametric tests.
§Only undetectable levels found in the IQR.
ACR, albumin to creatinine ratio; a-dsDNA, anti-double-stranded DNA; DMARD, disease-modifying antirheumatic drugs (detailed information is provided in online supplemental material); dt, detectable (detection limit was defined by ELISA manufacturer's recommendations as >9 pg/mL); eGFR, glomerular filtration rate; IL, interleukin; LN, lupus nephritis; M, mean; MES, mesangioproliferative lupus nephritis, class II or chronic inactive III; mg, miligram; MN, membranous lupus nephritis, class V; n-dt, non-detectable; p, plasma; PLN, proliferative lupus nephritis, International Society of Nephrology class III, IV+V; SLEDAI-2K, Systemic Lupus Erythematosus Disease Activity Index 2000; u, urine.

supplemental figure 1D). This was not observed in the MLN or MES group (data not shown). As activity index was generally low in MLN and MES, no comparison with u-IL-16 was made.

Discriminatory value of p-IL-16 and u-IL-16 measurements for PLN

Next, we investigated the hypothesis that u-IL-16 or p-IL-16 could serve as markers to discriminate PLN or any other subset of SLE. ROC and AUC were calculated for IL-16 in each biological fluid (figure 2A). u-IL-16 had the highest AUC value for discriminating PLN from all other groups (AUC=0.799, p<0.0001) (online supplemental figure 2A), while p-IL-16 did not demonstrate any significant predictive value in the model (online supplemental figure 2B).

We proceeded with investigating the performance of u-IL-16 and p-IL-16 in differentiating patients with LN

Table 3 Diagnostic value of the classic diagnostic parameters and detectable u-IL-16 for PLN

Parameters	Sensitivity	Specificity	PPV	NPV	P value*
PLN vs other active SLE					
Positive a-dsDNA, %	81.8	39.0	43.4	79.0	0.025
Low C3, %	78.4	71.3	55.8	87.7	<0.0001
Low C4, %	91.9	55.0	48.6	93.6	<0.0001
u-IL-16†, %	67.0	91.0	77.5	85.1	<0.0001
PLN vs other LN classes					
Positive a-dsDNA, %	81.8	51.4	66.0	63.3	0.012
Low C3, %	78.4	60.0	77.8	60.0	0.0082
Low C4, %	91.9	55.0	78.6	78.6	0.0002
u-IL-16†, %	67.0	83.8	83.3	66.0	<0.0001

*P value for two-tailed Fisher's exact test.
†The most optimal threshold value to calculate u-IL-16 sensitivity and specificity for PLN was determined as 15.8 pg/mL or higher, based on receiver operator characteristic curve analysis. Information on missing data is provided in the legend of tables 1 and 2.
a-dsDNA, anti-double-stranded DNA; LN, lupus nephritis; NPV, negative predictive value; PLN, proliferative lupus nephritis; PPV, positive predictive value; SLE, systemic lupus erythematosus; u-IL-16, urinary interleukin 16.

from patients with ANR-SLE using u-ACR as a control parameter. We confirmed that u-ACR effectively discriminated LN from other patients with active SLE (AUC u-ACR 0.963), while the AUC for u-IL-16 was 0.709 and for p-IL-16 the AUC not significant (figure 2A).

Next, we investigated the performance of u-IL-16 and p-IL-16 in differentiating PLN from other LN classes in a similar manner. Indeed, u-IL-16 performed best in this model (AUC 0.775, p=0.0001 for u-IL-16 and AUC 0.714, p=0.002 for u-ACR, respectively; figure 2B). Then, in order to investigate if PLN activity (active LN defined as activity index 3 or higher, n=42) affected the results, we repeated this analysis including only patients with active PLN, resulting in an AUC of 0.795 (p=0.0006) for u-IL-16, while the AUC for u-ACR was lower (0.703, p=0.004) (figure 2C). Lastly, we analysed if u-IL-16 differentiates active PLN from MLN, resulting in u-IL-16 AUC of 0.818 (p=0.002) and u-ACR AUC of 0.677 (p=0.02) (figure 2D).

Further, we investigated the performance of u-IL-16 in patients with significant albuminuria (here defined as u-ACR of >30 mg/mmol). u-IL-16 demonstrated a discriminatory value of AUC of 0.833 (p=0.0098), while the AUC for u-ACR was not significant (figure 2E). Finally, we investigated u-IL-16 in patients with LN with higher levels of albuminuria (u-ACR >50), finding an AUC of 0.896 (p=0.0096) for u-IL-16, while the AUC for u-ACR was not significant (figure 2F).

The AUC values for p-IL-16 were investigated in parallel in all these analyses but were not significant in all above cases.

Sensitivity and specificity of u-IL-16 for PLN

Next, we investigated the sensitivity and specificity of u-IL-16 for PLN in comparison with the classic LN biomarkers such as a-dsDNA, low C3 and C4, and albuminuria (u-ACR ≥30). As identified by the ROC curves, the most optimal cut-off for u-IL-16 to calculate the sensitivity and specificity was 15.8 pg/mL. u-IL-16 demonstrated a sensitivity of 67.0% for PLN with this cut-off value, which was lower than for the occurrence of a-dsDNA or low C3 or C4 levels (table 3). However, dt-u-IL-16 was found to have a high specificity of 91.0% for PLN among all patients with active SLE and 83.8% among all patients with LN (table 3). In online supplemental figure 2, we demonstrate the distribution and partial overlap of biomarkers among patients with PLN, where 13 of patients with other LN types did not display any of these biomarkers.

High proportion of kidney infiltrates contain cells expressing IL-16

Kidney biopsies acquired from 11 patients with LN were stained and evaluated for IL-16 expression (online supplemental table 2). We observed that variable proportions of the cells (median 55%, IQR 10–95%) within renal interstitial inflammatory infiltrates expressed cytoplasmic IL-16, while scarce or no staining was observed in glomerular cells (figure 3A–C). Numerically, untreated patients with PLN seemed to have more abundant infiltrates with higher proportion of IL-16 staining (figure 3A,B). On single CD3 staining, we observed that a higher proportion of infiltrating cells were positive for IL-16 than CD3.

DISCUSSION

In this study we found that detection of IL-16 in urine was associated with PLN and could discriminate PLN from other LN subtypes. Further, we observed kidney infiltrating cells expressing IL-16, suggesting a role in LN pathogenesis. In contrast, elevated p-IL-16 levels were associated with SLE disease activity in general, but had no diagnostic value for LN.

The most important finding of this study is that u-IL-16 is associated with PLN, where logistic regression analysis showed that u-IL-16 could distinguish patients with

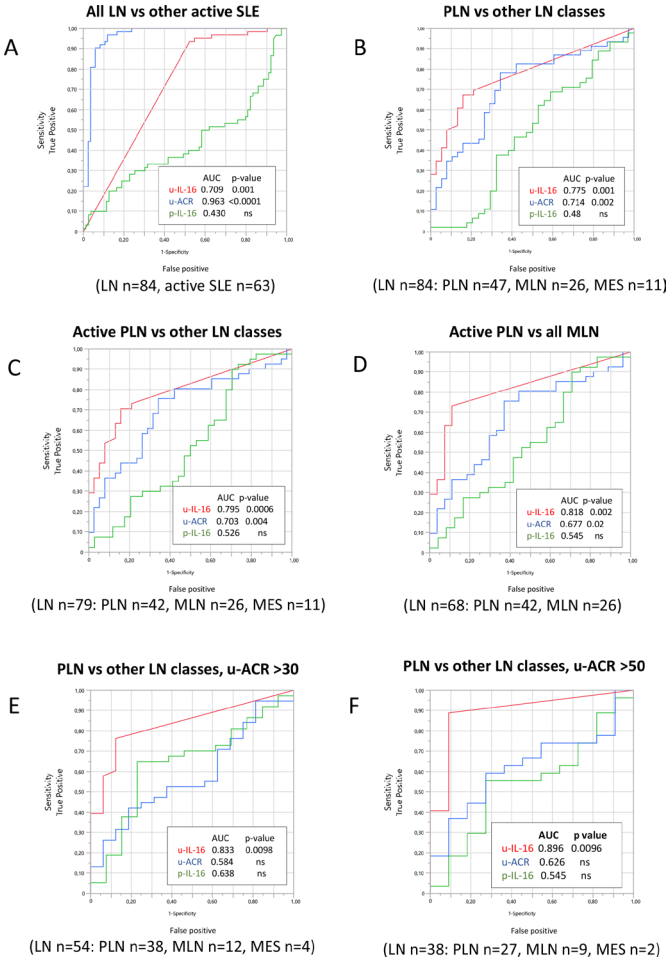

Figure 2 Logistic regression analysis of the diagnostic value of plasma (p-) and urinary (u-) IL-16 in subgroups of patients with LN. (A) ROC curves for u-IL-16, p-IL-16 and u-ACR demonstrate how these variables differentiate LN from ANR-SLE. (B) AUC demonstrates the diagnostic value of u-IL-16 and p-IL-16 for PLN compared with u-ACR among patients with other LN classes. (C) AUC demonstrates the diagnostic value of u-IL-16 and p-IL-16 for active PLN compared with u-ACR among patients with LN. (D) ROC analysis demonstrates the discriminative value of u-IL-16 and p-IL-16 in differentiating active PLN from MLN. (E) Discriminative value of u-IL-16 and p-IL-16 in differentiating PLN from other LN classes in patients with significant albuminuria of u-ACR >30 (corresponding to proteinuria of 500 mg/24 hours). (F) Discriminative value of u-IL-16 and p-IL-16 in differentiating PLN from other LN classes in patients with high albuminuria of u-ACR >50. ACR, albumin to creatinine ratio; ANR-SLE, active non-renal SLE; AUC, area under the curve; IL, interleukin 16; LN, lupus nephritis; MES, mesangioproliferative lupus nephritis; MLN, membranous lupus nephritis; PLN, proliferative lupus nephritis; ROC, receiver operator characteristic curve; SLE, systemic lupus erythematosus.

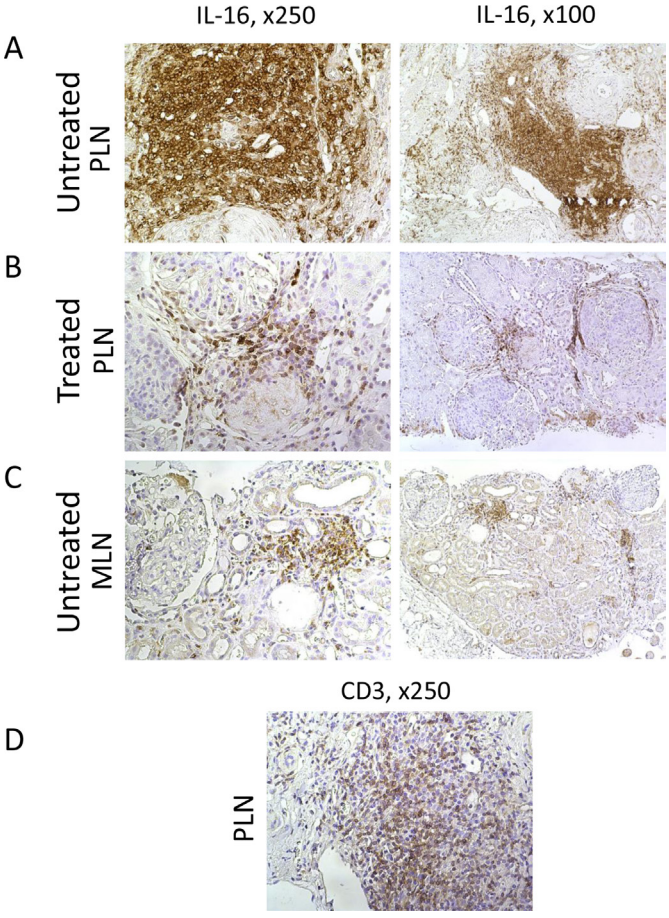

Figure 3 Expression of IL-16 and CD3 in LN kidney biopsies. (A) A high proportion of kidney infiltrating mononuclear cells express IL-16 in untreated PLN. (B) Numerically, a lower proportion of IL-16 positive cells were observed in patients with PLN with ongoing treatment (example of a patient on 10 mg of steroids). (C) A proportion of IL-16 positive kidney infiltrating cells were observed in MLN. (D) A proportion of the infiltrating cells express T cell marker CD3. IL-16, interleukin 16; LN, lupus nephritis; MLN, membranous lupus nephritis; PLN, proliferative lupus nephritis.

PLN from other LN subtypes, in particular patients with active PLN compared with patients with MLN. Of note, the discriminatory potential for PLN was highest among patients with LN with high levels of albuminuria.

Compared with high a-dsDNA titres and low C3 and C4, commonly used general biomarkers for SLE disease activity, and for LN in particular,[24] dt-u-IL-16 had a higher specificity for PLN as a marker of LN activity. The sensitivity for PLN on the other hand was slightly lower. Our findings thus suggest that the discriminatory potential of dt-u-IL-16 for PLN should be further investigated and validated. Our findings extend recent data reported by Fava *et al*,[10] who identified u-IL-16 as a marker of histological activity in PLN.

Previously, we and others have reported increased p-IL-16 levels in patients with active SLE, and particularly in patients with kidney involvement.[8 25 26] In this study, we confirm that patients with SLE with active disease have high circulating levels of IL-16, but this marker could not discriminate patients with renal or non-renal disease.

Among patients with PLN, u-IL-16 correlated moderately with renal activity index and degree of albuminuria, but the correlation was lost when patients with no signs of albuminuria were excluded. This goes in line with

findings by Fava *et al*, where no correlation with proteinuria was found.[10]

We found that more than half of the kidney infiltrating cells in LN express IL-16 protein. Numerically higher numbers of IL-16 positive cells were observed in patients without ongoing treatment. On CD3 staining, we observed a higher proportion of cells positive for IL-16 than CD3, indicating that other cells than T cells may express IL-16 in LN kidney. Similarly, variable proportions of positive cells were observed in both PLN and MLN. In contrast, u-IL-16 was only detected in a small proportion of MLN cases. The discrepancy between IL-16 expression at the tissue level in both PLN and MLN cases and pronounced IL-16 excretion in the urine in PLN remains to be investigated.

Type I interferons (mainly IFN-αs) have major importance in the pathogenesis of SLE.[27] The interplay between IFN-α and IL-16 has been described in systemic viral infections. As type I IFNs are difficult to measure, IL-16 has been suggested as an IFN-associated biomarker to identify a systemic viral inflammation.[28 29] It would be of interest if this could be used as a proxy of type I IFN signature and applied in patients with SLE since IL-16 detection is simple and can be performed at a limited cost. Importantly, it has been demonstrated that exposure of various cells to IFN-α may result in release of IL-16.[16 30] Thus, the interplay of IL-16 and IFNs is an interesting pathway to be further investigated in SLE systemically, and specifically in target organs, but was not further studied in this study.

It is not well studied how immunosuppressive treatments affect the production and secretion of IL-16. In our cross-sectional study, we could not draw any conclusions on the impact of ongoing treatment on IL-16 levels. Of note, being a real-life study cohort, several patients were under treatment with corticosteroids and/or ACE inhibitors and/or immune suppressants, which could have had affected the IL-16 levels and possibly also contributed to the relatively low albuminuria levels at the time of sampling.

Previous studies have shown that corticosteroids may suppress IL-16 levels.[31] Furthermore, a calcineurin-dependent downregulation of IL-16 mRNA has also been described in an animal model.[32] Voclosporin, a calcineurin inhibitor, is a promising new drug in the treatment of LN and therefore could be of interest to explore if and how it may modulate the expression of IL-16.[33 34]

The strength of our study is the well-characterised SLE cohort including four comparator groups and the large number of histopathologically classified LN cases, as well as IL-16 measurements in urine, plasma and kidney biopsies. The limitation is that some patients were recruited after the initiation of high-dosage steroid treatment and therefore we cannot exclude that treatment could have had an impact on the IL-16 levels. With the methods used, we could not identify the u-IL-16 sources nor determine if it was actively secreted or passively released.

In conclusion, our study contributes to the accumulating evidence of circulating IL-16 as an activity marker in SLE. More importantly, we have identified u-IL-16 as a novel discriminator between active renal and non-renal SLE, where u-IL-16 was found to associate with severe forms of PLN. Furthermore, the expression of IL-16 in renal tissue strongly suggests a role in LN pathogenesis. Thus, the IL-16 pathway is of interest for further investigation as a possible therapeutic target in severe forms of LN.

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Contributors IG, ES, TBN, AH and VO conceived the study. IG, ES, FF, AZ and VO recruited the patients and collected database and biological materials. AL, AH and VO performed the experiments. AH, IG, ES, AL, AZ, FF, TBN and VO interpreted and analysed the data. VO and AH drafted the manuscript. VO is responsible for the overall content. All authors participated in the manuscript revision and its final approval.

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Competing interests None declared.

Patient and public involvement Patients and/or the public were not involved in the design, or conduct, or reporting, or dissemination plans of this research.

Patient consent for publication Not required.

Ethics approval This study involves human participants and was approved by the Swedish Ethical Review Authority (2014/1337-31/3) and performed according to the Declaration of Helsinki. Participants gave informed consent to participate in the study before taking part.

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