SUPPLEMENTARY MATERIAL FOR:

Urinary markers differentially associate with kidney inflammatory activity and chronicity measures in patients with lupus nephritis

Ahmad Akhgar[1*], Dominic Sinibaldi[2], Lingmin Zeng[3**], Alton B. Farris III[4], Jason Cobb[5], Monica Battle[6], David Chain[7], Jennifer A. Cann[1**], Gábor G. Illei[8**], S. Sam Lim[9], Wendy I. White[10]

[1]Clinical Pharmacology and Safety Sciences R&D, AstraZeneca, Gaithersburg, Maryland, USA
[2]Applied Analytics and AI, BioPharmaceuticals R&D, AstraZeneca, Gaithersburg, Maryland, USA
[3]Late Oncology Biometrics, AstraZeneca, Gaithersburg, Maryland, USA
[4]Department of Pathology and Laboratory Medicine, Emory University Hospital, Atlanta, Georgia, USA
[5]Department of Medicine, Renal Medicine Division, Emory University, Atlanta, Georgia, USA
[6]Department of Rheumatology, School of Medicine, Emory University, Atlanta, Georgia, USA
[7]Clinical Proteomics, Translational Medicine, Early Oncology, AstraZeneca, Gaithersburg, Maryland, USA
[8]Clinical Development, Viela Bio, Gaithersburg, Maryland, USA
[9]Department of Medicine, Division of Rheumatology, Emory University, Atlanta, Georgia, USA
[10]Clinical Pharmacology and Quantitative Pharmacology, Clinical Pharmacology & Safety Sciences, BioPharmaceuticals R&D, AstraZeneca, Gaithersburg, Maryland, USA

[*]Current affiliation: Loxo Oncology at Lilly, New York City, New York, USA
[**]Affiliation during study conduct

Corresponding Author:
Wendy I. White
AstraZeneca
1 Medimmune Way
Gaithersburg, MD 20878
E-mail: wendy.white@astrazeneca.com

CONTENTS

SUPPLEMENTAL METHODS

Analyte Screening

To determine assay suitability and accuracy, dilutional linearity testing was performed by serially diluting the 9 urine samples at a ratio of 1:2 in 4% bovine serum albumin buffer from the minimum required dilution (MRD), which varied for each analyte. For an analyte to "pass" linearity testing, the back-calculated concentration of 50% of the diluted samples was required to recover within an average of ±30% of the MRD concentration. Analytes that had fewer than 3 participants passing linearity testing were excluded from further consideration.

Immunohistochemistry (IHC) Staining for Macrophage- and Neutrophil-associated Markers

Kidney biopsy sections measuring 2 μm from each paraffin block were cut and mounted on glass slides (Emory University histology lab) and shipped to AstraZeneca for staining. IHC staining was conducted using the Roche Ventana Discovery Ultra automated staining platform (Roche, Basel, Switzerland). Tissues were stained for CD163/myeloperoxidase (MPO) using a duplex IHC assay (Roche 760-4437; Roche 760-2659), matrix metalloproteinase-9 (MMP-9; Abcam ab76003) and Neutrophil Elastase (R&D MAB91671) using monoplex IHC assays. All IHC slides were digitally scanned using the Aperio AT2 and/or Aperio AT Turbo (Leica Biosystems, Concord, Ontario, Canada).

All slides were assessed for quality and suitability, and all evaluable biopsy tissue slides were analyzed using HALO®, a computer-assisted image analysis software (Indica Labs, Albuquerque, NM) to quantitate the density (number of cells/mm^2) or proportion (percent positive pixels) of each marker of interest specifically within the glomeruli and interstitial tissues

of the core needle kidney biopsies. All biopsies contained both cortex and medulla. With the pathologist's guidance (JAC), the glomeruli were identified by their distinct hematoxylin-stained morphology. The slides were manually annotated in HALO software by a single analyst to minimize variability for 4 layers: interstitium, healthy glomeruli, fibrotic glomeruli, and ambiguous glomeruli. The glomeruli within a slide were removed from further analysis if they were considered to be "ambiguous," such as if the glomerulus was partially present as a result of slicing on the edge of the tissue, had excessive sampling, or had staining artifacts. All glomeruli that did not fall into the "ambiguous" category, including "healthy" and "fibrotic," were counted together and included for analysis, regardless of the presence or degree of fibrosis. Tailored algorithms were developed to detect and quantify cells that were immunoreactive for CD163, neutrophil elastase, or both, in the glomerular and interstitial compartments, independently. The algorithms were optimized for specificity, sensitivity, and selectivity, and accuracy was confirmed via visual comparison of the original slides with the computer-generated cellular classifications for each core needle biopsy. For each core biopsy, the quantitative cell counts were normalized to the area of tissue analyzed and reported as "# + cells/mm^2" for each tissue compartment, glomeruli and interstitium, independently. Pairwise Wilcoxon tests were run for each IHC stain across each tissue category to determine statistically significant differences.

SUPPLEMENTARY TABLES

Supplementary Table S1. List of 112 analytes selected for inclusion in the primary analyte pool.

Analyte name
6Ckine
ADAMTS8
Adiponectin
Alpha-1-acid glycoprotein 1
Alpha-1-antitrypsin
Alpha-2-macroglobulin
Angiogenin
Angiotensin-converting enzyme
Antithrombin-III
Apolipoprotein
Apolipoprotein A-I
Apolipoprotein A-II
Apolipoprotein B
Apolipoprotein C-I
Apolipoprotein C-III
Apolipoprotein H
B-cell–activating factor
Beta-2-microglobulin
C-reactive protein
Cathepsin D
C-C motif chemokine 15
CD5 antigen-like
CD40 antigen
CD163
Clusterin
Complement C3
Cystatin-B
Cystatin-C
Dopamine beta-hydroxylase
E-selectin
EN-RAGE
Fatty acid–binding protein, adipocyte
Fatty acid–binding protein, heart
Fatty acid–binding protein, liver
Ferritin
Fetuin-A
Fibrinogen

Fibulin-1C
Ficolin-3
Folate receptor gamma
Growth/differentiation factor 15
Growth-regulated alpha protein
Haptoglobin
Heat-shock protein 70
Hemopexin
Hepatocyte growth factor
Hepsin
Immunoglobulin A
Immunoglobulin M
Insulin-like growth factor-binding protein 2
Intercellular adhesion molecule 1
Interferon alpha[a]
Interferon gamma induced protein 10
Interferon-inducible T-cell alpha chemoattractant
Interleukin-1 alpha
Interleukin-1 beta[a]
Interleukin-2 receptor alpha
Interleukin-6[a]
Interleukin-6 receptor
Interleukin-6 receptor subunit beta
Interleukin-8
Kidney injury molecule-1
Lactoferrin
Latency-associated peptide of transforming growth factor beta 1
Leucine-rich alpha-2-glycoprotein
Luteinizing hormone
Macrophage colony-stimulating factor 1
Macrophage inflammatory protein-1 beta
Macrophage-stimulating protein
Matrix metalloproteinase-3
Monocyte chemotactic protein 1
Monokine induced by gamma interferon
Myeloperoxidase
Myoglobin
Neuropilin-1
Neutrophil activating peptide 2
Neutrophil gelatinase-associated lipocalin
Omentin
Osteocalcin

Osteoprotegerin
Pancreatic polypeptide
Pancreatic secretory trypsin inhibitor
Pepsinogen I
Pigment epithelium derived factor
Protein DJ-1
Pulmonary and activation-regulated chemokine
Receptor tyrosine-protein kinase erbB-3
Resistin
Retinol-binding protein 4
Serotransferrin
Serum amyloid P-component
ST2
Stem cell factor
Superoxide dismutase 1, soluble
T-cell–specific protein RANTES
Thrombin-activatable fibrinolysis
Thyroxine-binding globulin
TIMP-1
TIMP-2
Transthyretin
TNF ligand superfamily member 13
TNF receptor 1
TNF receptor 2
Urokinase-type plasminogen activator receptor
Uteroglobin
Vascular cell adhesion molecule-1
Vascular endothelial growth factor
Vitamin D–binding protein
Vitamin K-dependent protein S
Vitronectin
von Willebrand factor
YKL-40

[a]SIMOA panel analyte.

ADAMTS8, a disintegrin and metalloproteinase with thrombospondin motifs 8; CD, cluster of differentiation; EN-RAGE, extracellular newly identified receptor for advanced glycation end products binding protein; Ig, immunoglobulin; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; SIMOA, single assay molecule array; ST2, suppression of tumorigenicity 2; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

Supplementary Table S2. Fold-change of urinary protein analytes with significantly (P<0.05) higher expression compared with HDs.

	Fold change		
Analyte	**LN**	**SLE**	**Biopsy control**
Analytes present uniquely in a single cohort			
Thyroxine-binding globulin	125.50	-	-
Serum amyloid P-component	30.63	-	-
Folate receptor gamma	16.58	-	-
ST2	9.26	-	-
Neuropilin-1	7.18	-	-
Clusterin	6.59	-	-
Dopamine beta-hydroxylase	6.58	-	-
Fibulin-1C	5.74	-	-
Heat-shock protein 70	4.88	-	-
Macrophage stimulating protein	3.59	-	-
E-selectin	2.94	-	-
TIMP-2	2.90	-	-
Macrophage inflammatory protein-1 beta	2.76	-	-
Apolipoprotein A	2.50	-	-
Interleukin-6 receptor	2.48	-	-
Interleukin-2 receptor alpha	2.39	-	-
Vascular endothelial growth factor	2.24	-	-
Intercellular adhesion molecule 1	-	3.07	-
Pepsinogen I	-	2.04	-
Osteocalcin	-	-	40.77
Neutrophil activating peptide 2	-	-	35.68
Analytes present in 2 cohorts			
Leucine-rich alpha-2-glycoprotein	65.35	4.27	-

Interferon alpha[a]	15.62	5.18	-
EN-RAGE	13.97	10.87	-
Adiponectin	11.16	6.19	-
Angiogenin	2.98	2.16	-
T-cell–specific protein RANTES	2.60	2.09	-
Fatty acid–binding protein, adipocyte	498.36	-	554.49
Vitronectin	302.76	-	68.89
Pigment epithelium derived factor	251.91	-	655.26
Serotransferrin	192.67	-	97.58
Apolipoprotein B	103.28	-	98.43
Retinol-binding protein 4	94.92	-	96.48
Transthyretin	72.75	-	77.99
Hemopexin	72.42	-	105.96
Apolipoprotein C-I	58.62	-	52.89
Apolipoprotein A-I	49.78	-	204.15
Ficolin 3	38.48	-	24.99
Superoxide dismutase 1, soluble	35.95	-	52.89
Fatty acid–binding protein, heart	33.35	-	27.90
Complement C3	32.27	-	19.34
CD163	31.80	-	6.04
Apolipoprotein A-II	31.12	-	69.83
Alpha-2-macroglobulin	29.66	-	7.81
Apolipoprotein C-III	22.81	-	46.53
Myeloperoxidase	17.01	-	20.07
Growth-regulated alpha protein	13.80	-	19.58
Lactoferrin	11.27	-	16.09
Pancreatic secretory trypsin inhibitor	11.00	-	9.98
Interleukin-1 beta[a]	8.73	-	25.93
Tumor necrosis factor ligand superfamily member 13	7.77	-	8.55

Omentin	7.55	-	11.69
Kidney injury molecule-1	7.09	-	4.77
Pancreatic polypeptide	7.02	-	23.27
Stem cell factor	6.17	-	9.92
Neutrophil gelatinase-associated lipocalin	6.08	-	7.99
Cystatin-B	5.80	-	7.76
Protein DJ-1	5.38	-	8.93
Insulin-like growth factor-binding protein 2	5.00	-	6.28
ADAMTS8	5.00	-	5.67
Pulmonary and activation-regulated chemokine	4.52	-	4.81
C-C motif chemokine 15	4.41	-	7.44
Latency-associated peptide of transforming growth factor beta 1	3.89	-	4.72
Interleukin-6 receptor subunit beta	2.86	-	2.48
Analytes present in 3 cohorts			
Immunoglobulin A	760.14	177.83	248.32
Myoglobin	409.00	1148.47	2549.59
Haptoglobin	341.36	74.98	153.54
Alpha-1-antitrypsin	339.44	54.05	572.82
Alpha-1-acid glycoprotein 1	199.38	40.98	95.85
Antithrombin-III	184.24	5.23	73.18
Vitamin D–binding protein	125.10	6.92	154.38
Ferritin	84.05	39.30	63.54
Beta-2-microglobulin	52.80	10.92	219.30
Vitamin K–dependent protein S	52.02	3.47	92.00
Fibrinogen	47.86	5.72	129.63
Fetuin-A	34.68	5.52	30.91
Uteroglobin	30.52	6.66	32.38
B-cell–activating factor	26.70	11.37	20.50
von Willebrand factor	21.59	5.85	5.81

Matrix metalloproteinase-3	16.80	4.77	15.65
TIMP-1	16.45	4.12	20.96
Interleukin-6[a]	14.85	7.01	7.61
Vascular cell adhesion molecule-1	14.25	7.72	14.94
Cathepsin D	12.27	3.60	15.19
Interleukin-8	11.64	6.85	8.38
Cystatin-C	10.87	1.76	14.56
Monocyte chemotactic protein 1	10.68	4.34	4.32
Osteoprotegerin	9.29	3.37	9.67
Apolipoprotein H	8.63	2.21	25.75
Urokinase-type plasminogen activator receptor	6.32	4.52	8.56
Tumor necrosis factor receptor 1	6.29	3.44	7.46
Tumor necrosis factor receptor 2	6.25	4.50	4.10
Growth/differentiation factor 15	6.23	3.82	5.50
CD5 antigen-like protein	5.27	2.01	4.32
Interferon gamma induced protein 10	4.56	4.12	4.75
Resistin	4.56	2.75	6.04
Macrophage colony-stimulating factor 1	4.07	4.71	4.83
CD40 antigen	3.40	2.55	4.16
Hepatocyte growth factor	2.53	1.84	2.49
6Ckine	2.20	2.62	2.24

[a]SIMOA panel analyte.

ADAMTS8, a disintegrin and metalloproteinase with thrombospondin motifs 8; CD, cluster of differentiation; EN-RAGE, extracellular newly identified receptor for advanced glycation end products binding protein; HD, healthy donor; LN, lupus nephritis; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; SIMOA, single assay molecule array; SLE, systemic lupus erythematosus; ST2, suppression of tumorigenicity 2; TIMP, tissue inhibitor of metalloproteinase.

Supplementary Table S3. Narrowed protein analyte pools determined for NIH-AI and NIH-CI.

NIH-AI analytes (30 total)	NIH-CI analytes (26 total)
6Ckine	Angiogenin
ADAMTS8	Apolipoprotein C-I
Alpha-2-macroglobulin	Beta-2-microglobulin
Apolipoprotein A-II	C-C motif chemokine 15
Apolipoprotein B	CD40 antigen
Cathepsin D	Fatty acid–binding protein, adipocyte
CD163	Fetuin-A
EN-RAGE	Fibulin-1C
Fatty acid-binding protein, liver	Hepatocyte growth factor
Ferritin	Intercellular adhesion molecule 1
Heat-shock protein 70	Interferon-inducible T-cell alpha chemoattractant
Insulin-like growth factor-binding protein 2	Kidney injury molecule-1
Interferon alpha	Luteinizing hormone
Interleukin-1 alpha	Macrophage inflammatory protein-1 beta
Interleukin-2 receptor alpha	Matrix metalloproteinase-3
Interleukin-6	Osteocalcin
Latency-associated peptide of transforming growth factor beta 1	Pancreatic polypeptide
Macrophage-stimulating protein	Protein DJ-1
Monocyte chemotactic protein 1	T-cell–specific protein RANTES
Myoglobin	TNF ligand superfamily member 13
Serotransferrin	TNF 1
ST2	Urokinase-type plasminogen activator receptor
Superoxide dismutase 1, soluble	
TIMP-1	
Vascular cell adhesion molecule-1	
von Willebrand factor	
Analytes common to both NIH-AI and NIH-CI	
B-cell–activating factor	
Dopamine beta-hydroxylase	
Interleukin-6 receptor subunit beta	
Osteoprotegerin	

ADAMTS8, a disintegrin and metalloproteinase with thrombospondin motifs 8; AI, Activity Index; CD, cluster of differentiation; CI, Chronicity Index; EN-RAGE, extracellular newly identified receptor for advanced glycation end products binding protein; NIH, National Institutes of Health; RANTES, regulated upon activation, normal T cell expressed and presumably secreted; ST2, suppression of tumorigenicity 2; TIMP, tissue inhibitor of metalloproteinase; TNF, tumor necrosis factor.

Supplementary Table S4. NIH-AI and NIH-CI top canonical pathways.

Index	Pathway name[a]	*P*-value	Overlap	%
NIH-AI	**LXR/RXR activation**	**2.66E−12**	**8/123**	**6.5**
	Glucocorticoid receptor signaling	**3.17E−12**	**12/581**	**2.1**
	Acute phase response signaling	**7.15E−11**	**8/185**	**4.3**
	Hepatic fibrosis/hepatic stellate cell activation	5.07E−16	11/194	5.7
	Role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis	2.08E−10	9/325	2.8
NIH-CI	**Granulocyte adhesion and diapedesis**	**2.33E−11**	**8/189**	**4.2**
	Agranulocyte adhesion and diapedesis	**2.95E−09**	**7/214**	**3.3**
	Pathogenesis of multiple sclerosis	**1.05E−07**	**3/9**	**33.3**
	Hepatic fibrosis/hepatic stellate cell activation	6.39E−08	6/194	3.1
	Role of macrophages, fibroblasts, and endothelial cells in rheumatoid arthritis	5.24E−08	7/325	2.2

[a]Pathways unique to each index are shown in bold.

AI, Activity Index; CI, Chronicity Index; LXR, liver X receptors; NIH, National Institutes of Health; RXR, retinoid X receptors.

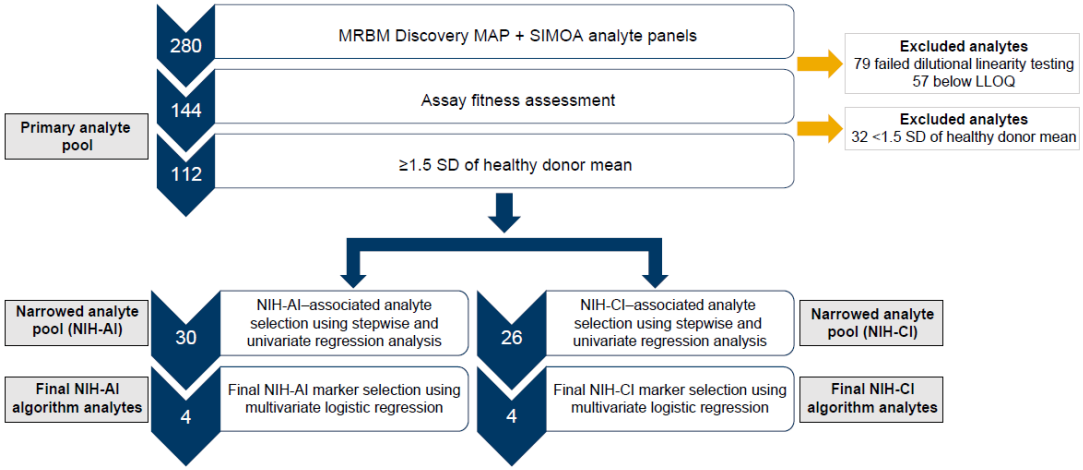
Supplementary Table S5. Analytes selected for use in predictive models

Index	Description
NIH-AI	Apolipoprotein A-II
	von Willebrand factor
	Interleukin-1 alpha
	Insulin-like growth factor-binding protein 2
NIH-CI	Dopamine beta-hydroxylase
	Kidney injury molecule 1
	Interleukin-6 receptor subunit beta
	Fetuin A

AI, Activity Index; CI, Chronicity Index; NIH, National Institutes of Health.

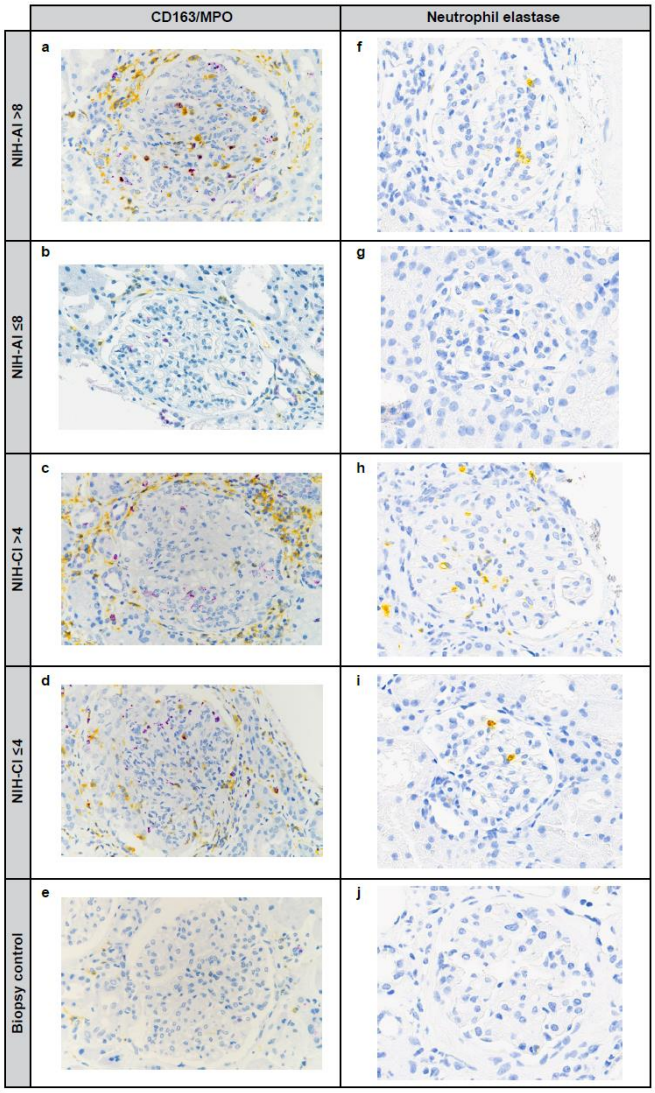
SUPPLEMENTARY FIGURES

Supplementary Figure S1. Protein analyte screening and selection workflow.



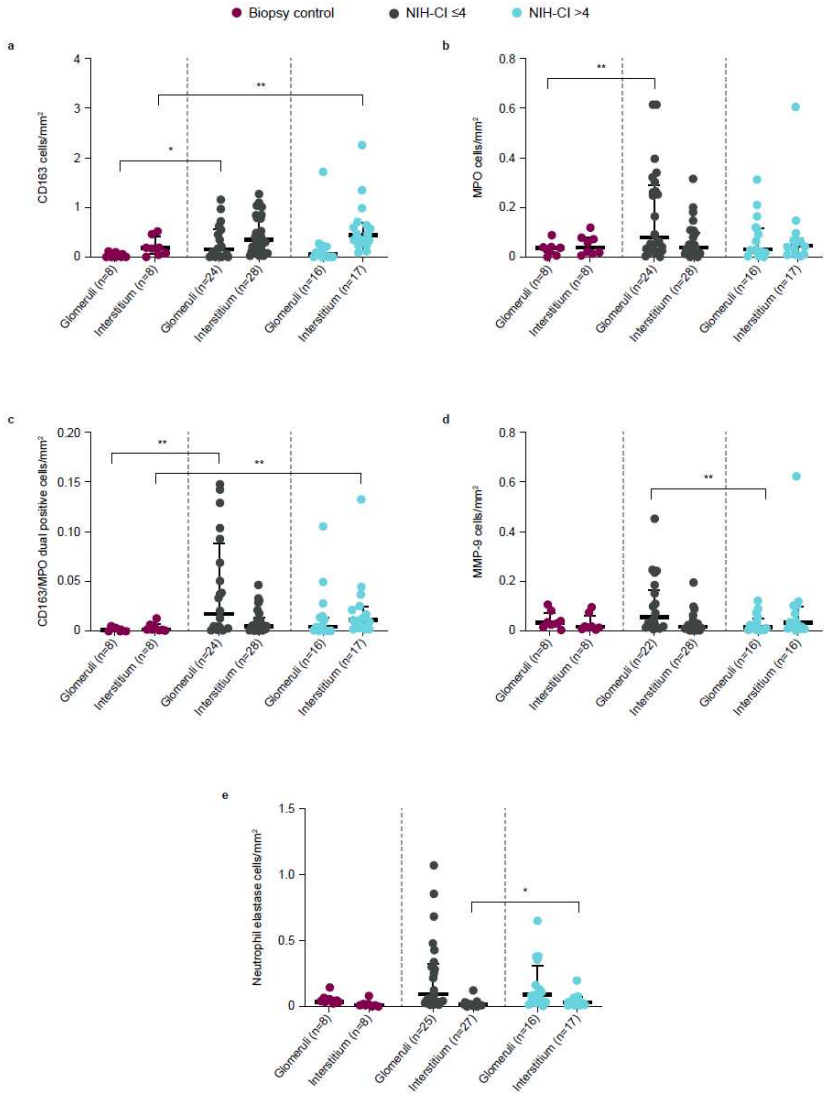
AI, Activity Index; CI, Chronicity Index; LLOQ, lower limit of quantification; MAP, Multi Analyte Panel; MRBM, Myriad Rules Based Medicine; NIH, National Institutes of Health; SD, standard deviation; SIMOA, single assay molecule array.

Supplementary Figure S2. Representative IHC staining of kidney biopsies from patients with LN compared with a biopsy control are shown. Macrophage-associated markers (CD163/MPO duplex, panels a–e, at 40× magnification); neutrophils (neutrophil elastase, panels f–j). Patients categorized as NIH-AI ≥8 (a and f), NIH-AI <8 (b and g), NIH-CI ≥4 (c and h), NIH-CI <4(d and i), and biopsy control (e and j) are shown.



AI, Activity Index; CD, cluster of differentiation; CI, Chronicity Index; IHC, immunohistochemistry; LN, lupus nephritis; MPO, myeloperoxidase; NIH, National Institutes of Health.

Supplementary Figure S3. Kidney biopsy IHC staining results from the NIH-CI ≥4 versus NIH-CI <4 patients with LN versus biopsy controls. Macrophage-associated markers: CD163/MPO duplex stain, MMP-9; neutrophil-associated marker: neutrophil elastase. Healthy and fibrotic glomeruli were counted together. (a) Number of CD163 positive cells/mm^2. (b) Number of MPO positive cells/mm^2. (c) Number of CD163/MPO dual positive cells/mm^2. (d) Number of MMP-9 positive cells/mm^2. (e) Number of neutrophil elastase positive cells/mm^2. **P*<0.01, ***P*<0.05.



CD, cluster of differentiation; CI, Chronicity Index; IHC, immunohistochemistry; LN, lupus nephritis; MMP-9, matrix metalloproteinase-9; MPO, myeloperoxidase; NIH, National Institutes of Health.