Supplementary Information

Soluble Mediators and Clinical Features Discern Risk of Transitioning to Classified Disease in Relatives of Systemic Lupus Erythematosus Patients

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Supplementary Patients and Methods

Study Population/Plasma Samples

Experiments were performed in accordance with the Helsinki Declaration and approved by the Oklahoma Medical Research Foundation (OMRF) and Medical University of South Carolina (MUSC) Institutional Review Boards. Blood relatives of lupus patients meeting < 4 American College of Rheumatology (ACR) SLE classification criteria(1, 2) (n=3645; mean time to follow-up = 8.0 years) previously enrolled in the Lupus Family Registry and Repository (LFRR) (3) or Systemic Lupus Erythematosus in Gullah Health (SLEIGH) study(4) (enrolled in the parent cohorts between 1992 and 2011) were invited by letter to participate in a follow-up study to identify individuals who transitioned to classified SLE (meeting $\geq$4 cumulative ACR criteria (1, 2)). Lupus relatives were recruited for the follow-up study between March 2010 and May 2012. Individuals who wished to participate in the study completed a short telephone interview to confirm their eligibility for the study, determine willingness to complete questionnaires and provide blood samples required for the study, and to screen for individuals who potentially met SLE classification criteria. All individuals who consented to this follow-up study (n=409) provided serum and plasma samples at the time of enrollment in the LFRR and SLEIGH studies, as well as this follow-up study, along with demographic and clinical information (3). Samples were stored at -20^{o}C and assays performed on freshly thawed samples.

Consent to obtain medical records was provided by study participants considered to potentially meet SLE classification criteria during the phone interview. Information regarding cumulative clinical and laboratory features for each case was obtained by chart review and collected on a standard data collection form by a rheumatology-trained physician or nurse. Clinical manifestations evaluated in this protocol were determined according to criteria set by the ACR (1, 2). Stringent documentation requirements were used for review of the medical record. Various levels of evidence were graded as follows: 0 = no evidence, 1 = patient-reported evidence, 2 = physician-reported evidence not confirmed by physical examination findings, and 3 = physician-observed evidence documented in the medical record. All designations used in this study had level 3 evidence. Each ACR criterion was recorded as being either present or absent. The date of occurrence and the presence or absence of each ACR criterion was recorded for each patient.

In addition to questionnaires to obtain demographic, education, socioeconomic, family pedigree, medical history, and medication data, participants completed the SLE-specific portion of the Connective Tissue Disease Screening Questionnaire (CSQ) (5, 6). The CSQ is a 30-item instrument designed to identify individuals with potential connective tissue disease in population studies and includes questions regarding symptoms of SLE, rheumatoid arthritis, scleroderma, mixed connective tissue disease (MCTD), myositis, and Sjögren's syndrome. The SLE portion of the CSQ (SLE-CSQ) was scored using an algorithm based on ACR classification criteria (5). The SLE-CSQ refers to nine criteria from the 1982 revised ACR criteria for SLE: malar rash, discoid rash, photosensitivity, oral ulcers, arthritis, serositis, proteinuria, hematologic disorder (anemia, leukopenia, low platelet count), and positive antinuclear antibody (ANA) titer. In addition, the SLE-CSQ refers to two criteria from the 1971 American Rheumatism Association criteria for SLE (alopecia and Raynaud's phenomenon). The CSQ instrument has been validated in community-based cohorts across multiple ethnicities (5-7).

Study participants who transitioned to classified SLE, meeting $\geq$4 cumulative ACR SLE classification criteria at follow-up (n=45) were demographically matched for case-control analysis by age ($\pm$5 years), gender, and race to non-transitioned study participants (1:2, 1 individual who transitioned to SLE matched to 1 ANA positive and 1 ANA negative unaffected

participant), as well as to unrelated healthy controls with no family history of SLE from the Oklahoma Immune Cohort (8) (1:1; 1 individual who transitioned matched to 1 control) who had data during the follow-up period. In addition to demographics and relationship to confirmed SLE patients, these matched groups were assessed for ACR criteria, SLE-CSQ scores, SLE-associated autoantibodies, and plasma soluble mediators in order to determine factors that differentiated relatives who transitioned to SLE.

Detection of SLE-associated Autoantibodies

Serum samples were screened for SLE-associated autoantibodies in OMRF's College of American Pathologists certified Clinical Immunology Laboratory, as previously described.(9) ANAs (HEp-2 cells) and anti-double-stranded DNA (anti-dsDNA by *Crithidia luciliae*) were measured using indirect immunofluorescence (Inova Diagnostics); a positive result was defined as detection of ANAs at a titer of $\geq$1:120 and anti-dsDNA antibodies at a titer of $\geq$1:30. Precipitin levels of autoantibodies directed against Ro/SSA, La/SSB, Sm, nRNP, and ribosomal P were detected by immunodiffusion. Anticardiolipin (aCL) antibodies were measured by enzyme linked immunosorbent assay, with a titer of >20 IgG or >20 IgM units considered positive.

In addition, plasma samples were screened for autoantibody specificities using the BioPlex 2200 multiplex system (Bio-Rad Technologies, Hercules, CA). The BioPlex 2200 ANA kit uses fluorescently dyed magnetic beads for simultaneous detection of 11 autoantibody specificity levels, including reactivity to dsDNA, chromatin, ribosomal P, Ro/SSA, La/SSB, Sm, the Sm/RNP complex, RNP, Scl-70, centromere B, and Jo-1.(9) Autoantibodies to dsDNA, chromatin, Ro/SSA, La/SSB, Sm, Sm/RNP complex, and RNP were used for analysis in the current study. Anti-dsDNA (IU/mL) has a previously determined positive cutoff of 10 IU/mL; an Antibody Index (AI) value (range 0-8) is reported by the manufacturer to reflect the fluorescence intensity of each of the other autoantibody specificities with a positive cutoff as AI=1.0. The AI scale is standardized relative to calibrators and control samples provided by the manufacturer.

Detection of Soluble Plasma Mediators

Plasma levels of BLyS (R&D Systems, Minneapolis, MN) and APRIL (eBioscience/ Affymetrix, San Diego, CA) were determined by enzyme-linked immunosorbent assay (ELISA), per the manufacturer protocol. An additional fifty analytes, including innate and adaptive cytokines, chemokines, and soluble TNFR superrelatives (appendix p 5), were assessed by xMAP multiplex assays (Panomics/Affymetrix, Santa Clara, CA).(10)

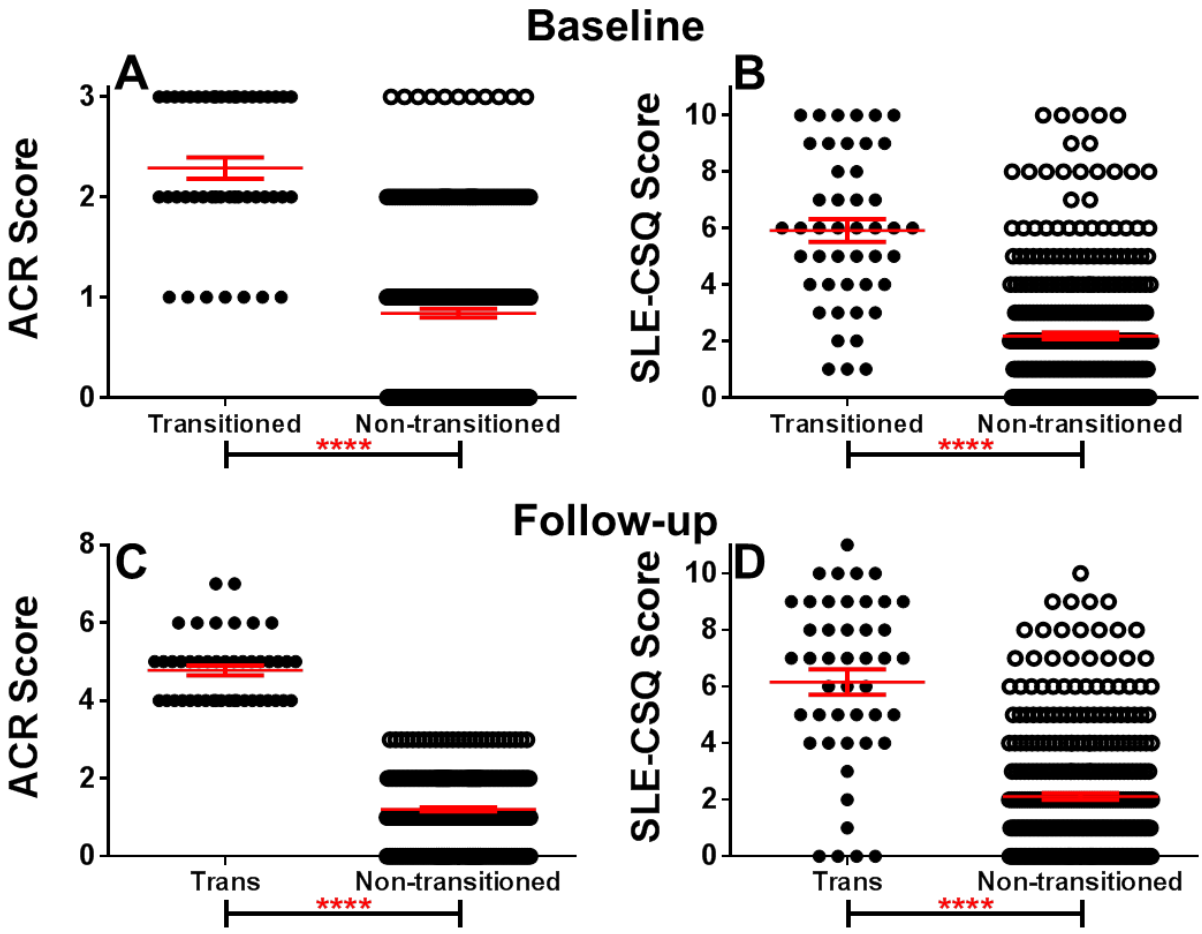
Data were analyzed on the Bio-Rad BioPlex 200® array system (Bio-Rad Technologies, Hercules, CA), with a lower boundary of 100 beads per analyte per sample. Median fluorescence intensity for each analyte was interpolated from 5-parameter logistic nonlinear regression standard curves. Analytes below the detection limit were assigned a value of 0.001 pg/mL. A known control serum was included on each plate (Cellgro human AB serum, Cat#2931949, L/N#M1016). Well-specific validity was assessed by AssayCheX™ QC microspheres (Radix BioSolutions, Georgetown, TX, USA) to evaluate non-specific binding. Mean inter-assay coefficient of variance (CV) of multiplexed bead-based assays for cytokine detection has previously been shown to be 10-14%,(11, 12) and a similar average CV (11%) was obtained across the analytes in this assay using healthy control serum. Intra-assay precision of duplicate wells averaged <10% CV in each 25-plex assay.

Statistical Analyses

Relatives who transitioned to classified SLE at follow-up were compared to non-transitioned relatives at baseline (pre-transition) and follow-up (post-transition). Chi-square or Fisher's exact test were used, as appropriate, to determine differences in gender, race, and familial relationship, as well as the presence of ACR criteria and lupus-associated autoantibody specificities, with Bonferroni adjusted p-values. Age differences were assessed by unpaired *t*-test with Welch's correction. Number of ACR criteria (ACR scores), SLE-CSQ scores, ANA titers, number of autoantibody specificities, and plasma soluble mediator levels were compared by Mann-Whitney test. Correlations between plasma soluble mediator levels and SLE-CSQ or ACR score were determined by Spearman rank correlation.

Differentiation modeling analyses were carried out in SAS version 9.3 (Cary, N.C.). The dataset was randomly split 2:1 into test and validation datasets. Generalized estimating equations (GEE), adjusting for correlation within families, were used to assess associations between demographic and familial factors, ACR scores, SLE-CSQ scores, and serology (autoantibody positivity and soluble mediators) at baseline and relatives who transitioned to classified SLE or remained unaffected at follow-up in the test dataset; the models were subsequently run in the validation dataset to determine how well they forecast previously unaffected relatives transitioning to SLE. Odds ratios were determined per 1 standard deviation change in soluble mediator level. The standard deviations of SCF and TGF-β were 329.2 and 105.2, respectively. GEE models were initially adjusted for age, gender, ethnicity, relationship to SLE patient, and ANA positivity at baseline (Base Model). Soluble mediators were then added as covariates to these models. Soluble mediators remained in the model if they were statistically significant ($p<0.05$). In addition, the effect of soluble mediators without ACR score and SLE-CSQ score was assessed. Receiver Operating Characteristic (ROC) curves were produced for each multivariable model in the test and validation datasets using transitioning to SLE as the outcome to determine goodness of fit. Area under the curve (AUC) was determined as the probability of concordance. Positive (LR+) and negative (LR-) likelihood ratios, as well as positive (PPV) and negative (NPV) predictive value were calculated utilizing the pre-test probability of transitioning to classified SLE = 11% (transition rate of this follow-up cohort) and the sensitivity and specificity of each of the GEE models in the test and validation datasets.

Supplementary Figures



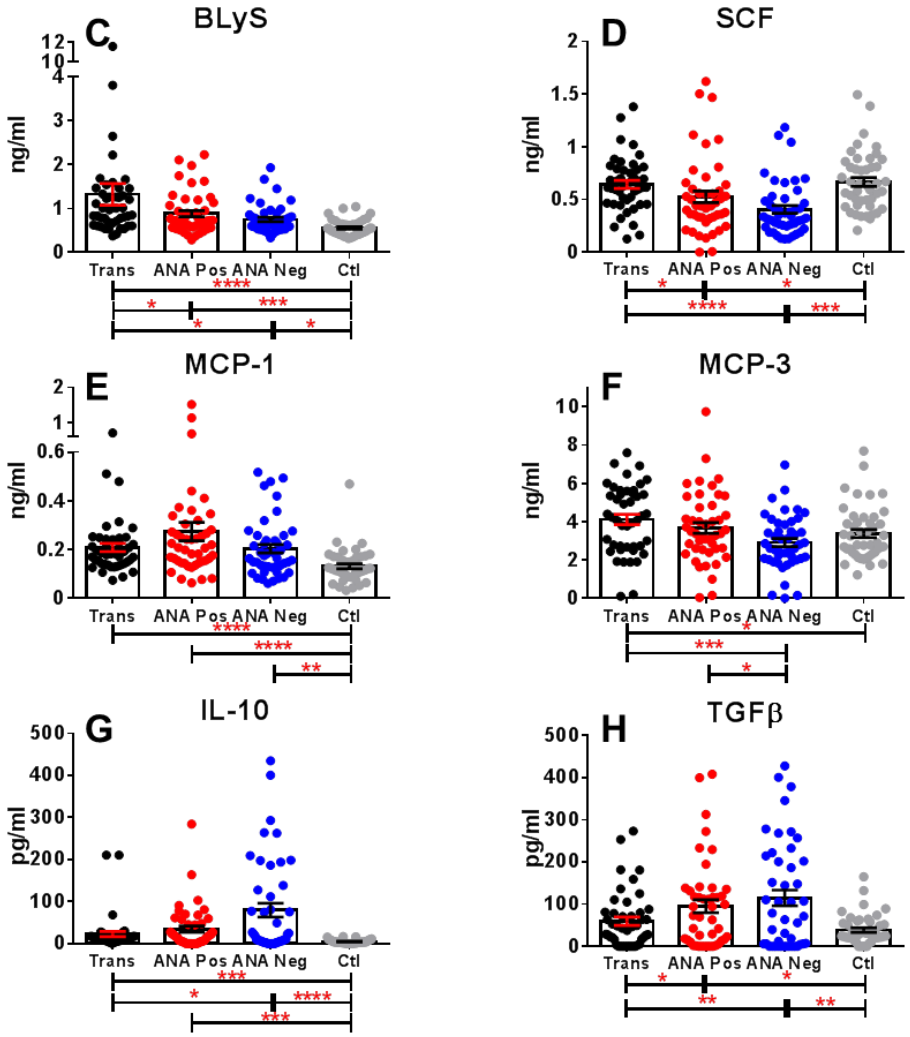
Supplementary Figure 1. Altered ACR and SLE-CSQ scores in relatives who transition to classified SLE. Baseline ACR (**A**) and SLE-CSQ (**B**) scores in 45 relatives who transitioned to classified SLE at follow-up (Trans) vs. 364 relatives who remained unaffected (Non-transitioned). Follow-up ACR (**C**) and SLE-CSQ (**D**) scores in 45 Trans vs. 364 Non-transitioned relatives. Mean ± SEM. ****$p<0.0001$ by Mann-Whitney.

A

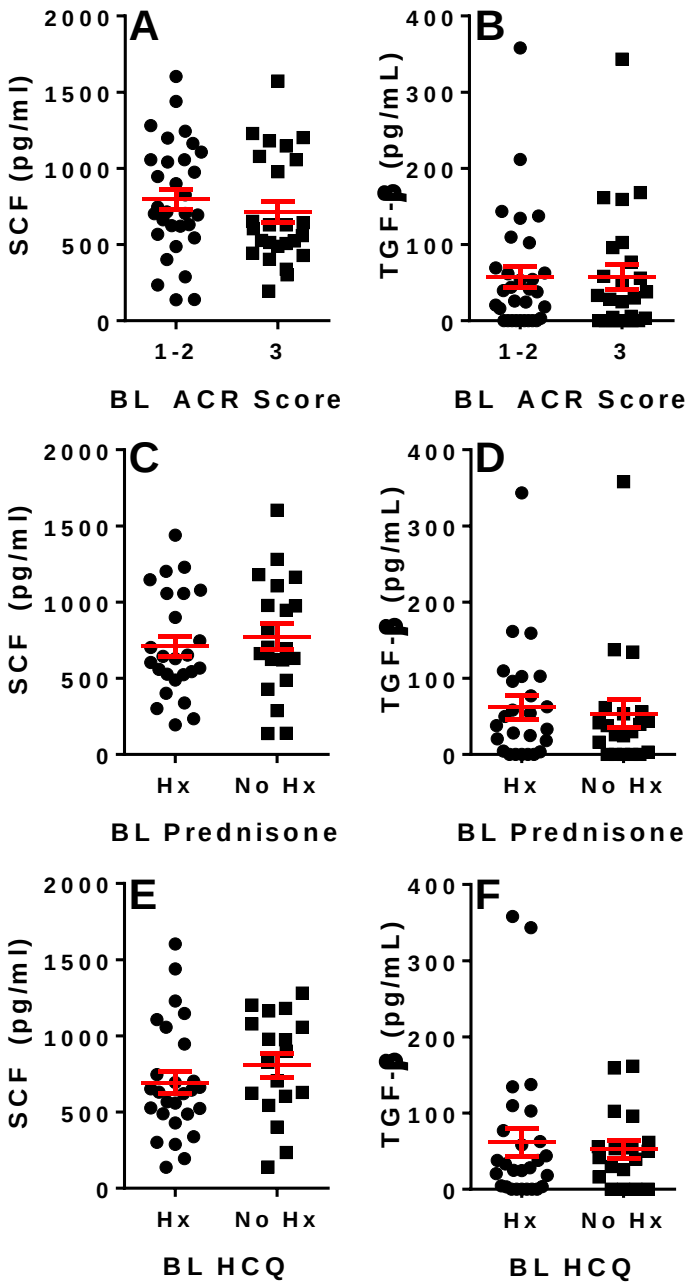
Biomarker	Spearman r	95% CI	p value
SLE-CSQ	0.528	0.390 to 0.643	***<0.0001***
SCF	0.341	0.179 to 0.487	***< 0.0001***
BLyS	0.254	0.084 to 0.411	***0.0028***
IL-10	-0.247	-0.404 to -0.765	***0.0039***
TGF-β	-0.238	-0.396 to -0.067	***0.0054***
MCP-1	0.212	0.040 to 0.372	***0.0135***
MCP-3	0.166	-0.009 to 0.330	*0.0550*
MIP-1β	0.074	-0.101 to 0.245	*0.3920*
TNFRII	0.030	-0.145 to 0.202	*0.7331*

B

Biomarker	Spearman r	95% CI	p value
ACR Score	0.528	0.390 to 0.643	***<0.0001***
SCF	0.356	0.194 to 0.499	***< 0.0001***
TNFRII	0.284	0.116 to 0.436	***0.0009***
MCP-3	0.277	0.108 to 0.430	***0.0011***
BLyS	0.265	0.096 to 0.420	***0.0019***
MIP-1β	0.230	0.059 to 0.389	***0.0072***
IL-10	-0.134	-0.301 to 0.041	*0.1216*
TGF-β	-0.100	-0.270 to 0.075	*0.2466*
MCP-1	-0.058	-0.117 to 0.229	*0.5070*



Supplementary Figure 2. Altered soluble mediators of inflammation in relatives who transition to classified SLE at follow-up. Spearman correlation of SLE-CSQ scores and plasma soluble mediator levels vs. ACR scores at follow-up are presented (**A**). Spearman correlation of ACR scores and plasma soluble mediators vs SLE-CSQ scores at follow-up are presented (**B**). Plasma levels of BLyS (**C**), SCF (**D**), MCP-1 (**E**), MCP-3 (**F**), IL-10 (**G**), and TGFβ (**H**) were measured at followup in 45 SLE who transitioned to classified SLE (Trans) vs. age (±5 years), race, gender, and time of sample procurement matched non-transitioned relatives who were ANA^+ (ANA Pos) or ANA^- (ANA Neg) by IIF, as well as to unrelated healthy controls (Ctl) with no family history of SLE. Mean ± SEM. ****p<0.0001; ***p<0.001; **p<0.01; *p<0.05 by Kruskal-Wallis with Dunn's multiple comparison.



Supplementary Figure 3. No difference in baseline SCF and TGF-β levels by ACR score or medication use in relatives who transition to classified SLE. Baseline plasma levels of SCF (**A, C, E**) and TGF-β (**B, D, F**) in 45 relatives who transitioned to classified SLE at follow-up. **A-B**. Transitioned relatives meeting 1-2 ACR criteria for SLE at baseline (BL) were compared to transitioned relatives meeting 3 ACR criteria for SLE at BL (ACR Score). Transitioned relatives with history (Hx) vs. no Hx at BL for prednisone (**C-D**) or hydroxychloroquine (HCQ, **E-F**) were compared. Mean ± SEM. *n.s.* by Mann-Whitney test.

Supplementary Tables

Supplementary Table 1. Plasma soluble mediators tested

Innate	Th1 associated	NGF/TNFR Superfamily
IL-1α	IL-12 (p70)	BLyS*
IL-1β	IFN-γ	APRIL*
IL-1RA	IL-2	sCD40L
IFN-α	IL-2RA	sFas
IFN-β		sFasL
G-CSF	Th17 associated	TNF-α
	IL-6	TNFRI (p55)
Homeostasis	IL-23	TNFRII (p75)
IL-7	IL-17A	TRAIL
IL-15	IL-21	NGFβ
Other	Th2 associated	Chemokine/Adhesion molecules
LIF	IL-4	IL-8/CXCL8
PAI-1	IL-5	IP-10/CXCL10
PDGF-BB	IL-13	RANTES/CCL5
Resistin		MIP-1α/CCL3
Leptin	Regulatory	MIP-1β/CCL4
SCF	IL-10	MCP-1/CCL2
	TGF-β	MCP-3/CCL7
		GROα/CXCL1
		SDF-1/CXCL12
		MIG/CXCL9
		Eotaxin/CCL11
		ICAM-1
		VCAM-1
		sE-selectin
*assessed by ELISA		VEGF-A

Supplementary Table 2. Frequency of Lupus Relatives

			Non-transitioned Matched to Transitioned to SLE[a]	
SLE Patients in Pedigree	**Non-transitioned**	**Transitioned**	**ANA Positive**	**ANA Negative**
1	228 (63%)	27 (60%)	27 (60%)	29 (64%)
2	94 (26%)	15 (33%)	14 (31%)	11 (24%)
3	25 (7%)	2 (5%)	2 (5%)	3 (7%)
4	9 (2%)	1 (2%)	1 (2%)	2 (5%)
5	8 (2%)	0	1 (2%)	0
Total	364	45	45	45

[a]ANA Positive and ANA negative (determined by IIF, titer ≥ 120) Non-transitioned relatives matched to Transitioned to SLE group by race, gender, and age (± 5 years)

Supplementary Table 3. Autoantibodies in Non-transitioned Relatives vs. Relatives Who Transition to SLE

	Non-transitioned		Transitioned		
Baseline	**(n=364)**		**(n=45)**		
ANA titer	**Median**	**IQR**	**Median**	**IQR**	***p-value*** [a]
	40	0-320	360	80-1080	***<0.0001***
# AutoAbs	**Mean**	**SD**	**Mean**	**SD**	***p-value*** [a]
	0.30	0.67	0.84	1.07	***<0.0001***
Specificities	**n**	**%**	**n**	**%**	***p-value*** [b]
anti-dsDNA	9	2.5%	5	11%	*0.0121*
anti-Ro/SSA	28	7.7%	12	27%	***0.0004***
anti-La/SSB	14	3.8%	6	13%	*0.0150*
anti-Sm	1	0.3%	1	2.2%	*0.2082*
anti-SmRNP	7	1.9%	2	4.4%	*0.2595*
anti-nRNP	8	2.2%	6	13%	***0.0020***
Follow-up					
ANA titer	**Median**	**IQR**	**Median**	**IQR**	***p-value*** [a]
	120	0-360	120	80-1080	***0.0007***
# AutoAbs	**Mean**	**SD**	**Mean**	**SD**	***p-value*** [a]
	0.23	0.60	0.78	1.06	***<0.0001***
Specificities	**n**	**%**	**n**	**%**	***p-value*** [b]
anti-dsDNA	12	3.3%	3	6.7%	*0.2215*
anti-Ro/SSA	33	9%	13	29%	***0.0004***
anti-La/SSB	13	3.6%	6	13%	*0.0114*
anti-Sm	1	0.3%	1	2.2%	*0.2082*
anti-SmRNP	8	2.2%	4	8.9%	*0.0328*
anti-nRNP	10	2.7%	4	8.9%	*0.0562*

[a]Mann-Whitney test

[b]Fisher's exact test; Bonferroni-adjusted p-value significance for multiple comparisons is *0.0071*

Supplementary References

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