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MEASUREMENTS OF CELL BOUND COMPLEMENT ACTIVATION PRODUCTS ENHANCE DIAGNOSTIC PERFORMANCE IN SYSTEMIC LUPUS ERYTHEMATOSUS

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ABSTRACT

Objective: To establish the value of cell-bound complement activation products in combination with antinuclear (ANA), anti-double-stranded DNA (anti-dsDNA) and anti-mutated citrullinated vimentin (anti-MCV) antibodies for the diagnosis of systemic lupus erythematosus (SLE).

Methods: The study was multicentred, cross-sectional and enrolled 593 patients (210 SLE patients, 178 patients with other rheumatic diseases and 205 healthy subjects). Complement receptor 1 levels on erythrocytes (ECR1) together with complement C4d levels on erythrocytes (EC4d), platelets (PC4d), and B-cells (BC4d) were determined using fluorescence-activated cell sorting. Serological markers were measured using enzyme-linked immunosorbent assays. Statistical analyses utilized area under receiver operating characteristic (ROC) curves, logistic regression, and calculations of diagnostic sensitivity and specificity.

Results: Anti-dsDNA was an insensitive (30%) but specific (>95%) marker for SLE. Several-fold higher EC4d, BC4d, PC4d and lower ECR1 were observed in SLE compared to other rheumatic diseases and healthy subjects. Among 523 anti-dsDNA negative subjects, multivariate logistic regression analysis revealed that SLE was associated with ANA positivity (≥20units), anti-MCV negativity (<70units) and elevation of both EC4d and BC4d ($p<0.001$) (ROC area=0.918). A positive Index score corresponding to the weighted sum of these four markers correctly categorized 72% of SLE patients. Specificity against other rheumatic diseases and normal healthy controls was >90%. Altogether, the combination of anti-dsDNA and the Index score positivity yielded 80% sensitivity for SLE, and 87% specificity against other rheumatic diseases.

Conclusion: An assay panel combining anti-dsDNA, ANA, anti-MCV, EC4d and BC4d is sensitive and specific for the diagnosis of SLE.

INTRODUCTION

Systemic lupus erythematosus (SLE) is a chronic autoimmune disease resulting in autoantibody-mediated tissue damages and potentially life-threatening multi-organ failure (1). This heterogeneous inflammatory disease affects from 161,000 to 322,000 adults in the United States with females being affected 9 times more often than men (2). In addition, the prevalence rate is higher in African Americans and Hispanics compared to Caucasians and socio-demographic background is predictive of poor prognosis (3). The manifestations of SLE are diverse and include rash, arthritis, anemia, thrombocytopenia, serositis, nephritis, seizures, and psychosis. Because these symptoms are heterogeneous, non specific, evolutive, and often mimic those of other diseases, the diagnosis of SLE is complex and can be challenging to physicians. Diagnosing SLE relies on a combination of the patient's medical history, current symptoms, and laboratory tests. Although the revised American College of Rheumatology criteria published in 1982 (4) requires the presence of 4 of 11 criteria to classify a patient as having SLE, the use of these criteria in clinical practice is not uniform (5).

Among the standard laboratory tests commonly used to support the diagnosis of SLE are primarily antinuclear antibodies (ANA) and anti-double stranded DNA (anti-dsDNA) antibodies (6). Nonetheless, ANA and anti-dsDNA antibodies have limitations and neither of these serological markers provides adequate balanced sensitivity and/or specificity to diagnose SLE. It is well recognized that complement activation is central to the pathogenesis of SLE (7), and a decade of biomarker research has illustrated the potential usefulness of cell bound complement activation products (CB-CAPs) to facilitate the diagnosis of SLE (8;9). These CB-CAPs include complement C4 derived ligand deposited on erythrocytes (EC4d)(8;10), platelets (PC4d)(9) and B lymphocytes (BC4d) (11) and their relative increase in SLE compared to other diseases may be of diagnostic value. In addition, SLE patients have reduced levels of the CR1 receptor (CD35)(8) on erythrocytes (ECR1). Consequently, there is a decrease in the clearance of immune complexes with an increased likelihood of accumulation at sites such as the kidney. It follows that

measurements of C4d deposition on cells together with determination of ECR1 densities may improve the performance of SLE diagnostics over conventional serologic profiling (e.g. ANA, anti-dsDNA). In addition, reports have established the potential contribution of CB-CAPs to disease activity (12;13) and their measurement may help treating physicians with the management of SLE patients. In the present study we have evaluated the contribution of CB-CAPs to the differential diagnosis of SLE. Our data indicate that an assay panel combining ANA and anti-dsDNA together with EC4d and BC4d offers an SLE diagnostic with enhanced sensitivity and specificity.

METHODS

Patients and study protocol

The study of complement activation products in the assessment of lupus (CAPITAL) was multi-centered, cross-sectional and required one, or at most two subject visits for screening and blood sample collection. There were no follow-up visits required. The study enrolled adult patients (≥18years) who were classified as having SLE according to the 1982 American College of Rheumatology classification criteria (4) updated in 1997 (14), patients with other well defined rheumatic diseases and normal healthy volunteers. All patients with rheumatic diseases were enrolled as part of their routine care. After the subject's informed consent was obtained the subject's medical history related to the diagnosis of rheumatologic conditions was obtained and reviewed for inclusion/exclusion criteria. Disease activity was measured at the time of the study visit using the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA) version of the SLE Disease Activity Index (SLEDAI) in all SLE patients(15). Active disease was defined as a SELENA-SLEDAI score ≥6 (16). The study was approved by an internal review board at each site and all patients provided informed consent. Blood was collected in EDTA-containing tubes for analysis of CB-CAPs, and serum was collected for the analysis of autoantibodies. The blood was shipped overnight from

the participating center to our remote clinical laboratory (San Diego, CA). CB-CAPs were analyzed by fluorescence-activated cell sorting (FACS) within 24 hours of receipt in the clinical laboratory. Serum was stored at -80°C prior to enzyme linked immuno-sorbent assay (ELISA) measurements. All laboratory scientists in charge of the FACS and ELISA assays were blinded to the patients' diagnoses throughout the study.

FACS measurements

C4d deposited on erythrocytes (EC4d), B lymphocytes (BC4d), platelets (PC4d) and CR1 expressed on erythrocytes (ECR1) were measured using a validated FACS assay.

EC4d and ECR1: whole blood (50µl) was washed with Dulbecco's phosphate buffered saline, centrifuged for 5 minutes (800g) and erythrocyte pellets were resuspended with 500µl of 1% normal goat serum solution (Jackson Immunoresearch Laboratories, West Grove, PA). A 10µl erythrocyte suspension was subsequently stained with purified mouse monoclonal antibodies against human C4d (mouse anti human C4d, Quidel inc, San Diego), human CR1 (mouse anti-human antibody produced by Taconic Biotechnology, Hudson, NY), or alternatively using non-specific mouse anti-human IgG1 kappa antibody (MOPC-21, BD Biosciences, San Jose, CA) for 45 minutes at 4°C. Samples were then washed as described above. Erythrocyte pellets were re-suspended in a solution (25 µl) containing goat anti-mouse antibody conjugated to fluorescein isothiocyanate (FITC, Jackson ImmunoResearch Laboratories, West Grove, PA) for 45 minutes at 4°C (in the dark). Following staining, washing and resuspension with 250µL of cold 1% normal goat serum solution the erythrocytes were subjected to FACS analysis for detection of C4d or CR1 on the cell surface.

BC4d levels: following lysis of erythrocytes from whole blood (700µl) using ammonium chloride-based reagent (BD Pharm Lyse, BD Bioscience, San Jose, CA) and centrifugation (5 minutes at 800g), cell pellets were resuspended in 500µl of a 1% normal goat serum solution and stained using monoclonal C4d antibody

(45 minutes at 2-8°C) as described above. A 25 µl cell suspension was subsequently stained using purified mouse monoclonal antibodies against human C4d or non-specific mouse anti-human IgG1 kappa antibody as above for 45 minutes at 4°C. Cell surface C4d staining was detected using goat anti-mouse fluorescein isothiocyanate (FITC) antibody (45 minutes at 2-8°C, dark). A monoclonal antibody against human CD-19 (a 95 kDa type I transmembrane glycoprotein expressed during all stages of B-cell differentiation and maturation) conjugated to R-phycoerythrin (R-PE) was used to detect the C4d complement activation derived fragment specific to the B-lymphocytes.

PC4d levels: platelet cells obtained from patient whole blood samples were tested using the C4d monoclonal antibody to measure cell surface levels of C4d by FACS as above. Whole blood samples (50µl) were diluted and stained with the monoclonal antibody against human C4d (45 minutes at 2-8ºC), followed by staining with goat anti-mouse conjugated to FITC (45 minutes at 2-8°C, dark). A monoclonal antibody against human CD42b conjugated to R-PE was used to identify the C4d complement activation derived fragment specific to the platelets.

All FACS analyses used a Beckman Coulter FC500 cytometer and CXP software (Beckman coulter, Brea CA). The mean fluorescence intensity (MFI) for the isotype background control and each complement protein (C4d, CR1) was obtained, and the net MFI was then determined by subtracting the non-specific MFI from the specific MFI results. Median inter-day (5 consecutive days) coefficient of variations for EC4d levels at low, medium and high intensity were established using blood from 44 patients with rheumatic diseases and ranged from 3.3 to 9.6% for EC4d and from 4.4 to 4.9% for ECR1. For BC4d inter-day coefficient of variations at low, medium and high intensity in 34 patients with rheumatic diseases ranged from 5.3 to 12.1%. Inter-day (5 consecutive days) coefficient of variations for PC4d in 12 patients with rheumatic diseases was 15.7%. In all FACS experiments controls establishing proper calibration, compensation and linearity of the flow cytometer were included.

ELISA measurements

ANA, anti-dsDNA and anti-mutated citrullinated vimentin antibodies [anti-MCV, an anticitrullinated peptide antibody])(17) were measured using enzyme linked immuno-sorbent assays (ELISA). All ELISA methods used have been cleared by the US Food and Drug Administration as safe and effective for in vitro diagnostic uses. ANA and anti-dsDNA were from INOVA Diagnostics (INOVA, San Diego, CA) and anti-MCV was from Orgentec Diagnostika, Germany). Intra-day and inter-day coefficient of variations for all methods were established in our clinical laboratory and were below 20%. For all ELISA experiments appropriate positive and negative controls were included.

Statistical analysis

Statistical analysis was conducted using the R software. Receiver operating curves were used as appropriate (18) for each of the markers (univariate analysis) and also following the determination of an index value as the output of multivariate logistic regression equation. As measures of performance, sensitivity and specificity (1- false positives rate) were computed. Confidence intervals were calculated by estimating the asymptotic standard error for a binomial proportion; these were confirmed using the Agresti-Coull confidence interval (19), with very similar results (within 1%; data not shown). Group comparisons were performed using Mann-Whitney or χ^2 tests as appropriate. Analyses evaluating the contributions of CB-CAPS to disease activity (SLENA-SLEDAI) were exploratory.

The reported performance statistics (sensitivity, specificity, ROC AUC) were calculated using apparent validation, also known as re-substitution validation, in which model performance is assessed directly from the samples used to derive the model. This validation strategy is known to give a biased or optimistic estimate of model performance. Therefore, the size of this bias was estimated using a bootstrapping procedure to quantify the optimism correction and obtain an "optimism corrected performance estimate" (20). Using this resampling procedure, with 5000 sampling draws, the optimism bias for sensitivity and

specificity of the models (both with and without anti-dsDNA) was consistently less than 1% thereby indicating that the performance estimates calculated using apparent validation were very close to the more complex optimism corrected performance estimates.

RESULTS

A total of 593 individuals were enrolled at 14 participating sites across the United States from April to August 2010. All patients gave informed consent. This consisted of 210 SLE patients (90.5% females, mean age 41y), 178 patients with other rheumatic diseases (80.3% females, mean age 57y, 63% caucasians), and 205 healthy individuals (65.9% females, mean age 41y, 56% Caucasians). Characteristics for the 210 SLE patients are presented in **Table I**. Disease activity scores were assessed using the SELENA-SLEDAI and were available in 209 SLE patients. The group of 178 patients with other rheumatic diseases included rheumatoid arthritis (RA, 120 patients), systemic sclerosis (21 patients), dermatomyositis (9 patients), vasculitis (8 patients), Sjogren's Syndrome (8 patients), polymyositis (7 patients), Granulomatosis with Polyangiitis (previously known as Wegener's Granulomatosis) (2 patients), fibromyalgia (two patients) and Sjogren with fibromyalgia (one patient).

Contribution of serological and CB-CAPs biomarkers by univariate analysis

Serological markers (anti-dsDNA, ANA, anti-MCV) and CB-CAPs were determined in all individuals (n=593). Using manufacturer cutoffs anti-dsDNA was insensitive (29.5%, 62 SLE patient positives) yet specific against other rheumatic diseases (96.1%; 7 false positive patients, 6 with RA and one with systemic sclerosis) and normal healthy individuals (99.5%, one false positive individual positive). In contrast, ANA was a highly sensitive marker (89% of SLE patients tested positives) yet largely non-specific against other

rheumatic diseases (59.0%). ANA specificity against normal healthy individuals was 90.7%. Results are presented in **Table II**.

CB-CAPs analysis revealed that SLE patients had several-fold higher C4d levels deposited on erythrocytes (2.8-fold), B-lymphocytes (3.2-fold) and platelets (4.5-fold) than patients with other rheumatic diseases ($p<0.001$). Several fold higher C4d levels results were also observed in SLE compared to normal healthy volunteers ($p<0.001$). SLE patients had lower CR1 levels on erythrocytes compared with patients with other diseases or normal subjects ($p<0.01$). ROC analysis comparing SLE and other diseases revealed that EC4d (AUC= 0.825) and BC4d (AUC=0.822) were the best predictors followed by PC4d (AUC=0.739) and ECR1 (AUC= 0.625). **Table II** highlights the performances characteristics of CB-CAPs at optimal cutoffs with sensitivity ranging from 46.2% (PC4d) to 70.0% (EC4d) and specificity (ranging from 48.9% (ECR1) to 92.7% (PC4d). Among the 205 normal healthy individuals, specificity for CB-CAPS was above 90% with the exception of ECR1 (69.8%).

Contribution of serological and CB-CAPs biomarkers by multivariate analysis

Because dsDNA positivity was highly specific for SLE (>95%), we next evaluated the predictive values of CB-CAPs, ANA, anti-MCV and their capacity to improve diagnostic sensitivity while maintaining adequate specificity (low false positive rates). Among 523 anti-dsDNA negative individuals comprised of 148 SLE patients, 171 patients with other diseases and 204 normal healthy controls, multivariate logistic regression analysis revealed that the stepwise addition of ANA positivity (ANA≥20 units, $p<0.001$), log normalized EC4d and BC4d net MFI ($p<0.001$) together with anti-MCV negativity (positivity for anti-MCV>70 is affected with a negative coefficient, $p<0.001$) increased the ROC AUC from 0.808 to 0.918. (**Table III**). ECR1 and PC4d did not significantly contribute to the model ($p>0.10$). The best model corresponded to an Index score of weighted sum of these four markers (estimates and Odds Ratio are provided in **Table IV**) was 1.20 (CI95%: 0.86; 1.53) in SLE, -2.54 (CI95%: -2.83;-2.24) in other rheumatic diseases, and -2.74 (CI95%: -2.89;-2.59) in normal healthy subjects (**Figure 1A**). Using an index cutoff at zero, sensitivity

among anti-dsDNA negative SLE patients was 71.6% (106/148)(CI95%: 64.4%;78,9%), specificity among anti-dsDNA negative patients with other diseases was 90.1% (154/171)(CI95%: 85.6%;94.5%) and specificity among dsDNA negative normal healthy was 98.0% (200/204)(CI95%: 96.1%;99.9%) (**Table IV**). **Figure 1B** illustrates the index values and the sensitivity and specificity at various index scores.

Altogether, the combination of anti-dsDNA and Index score positivity yielded 80.0% sensitivity (CI95%: 74.6%;85.4%) for SLE (168/210 SLE, including 62 anti-dsDNA positive patients and 106 patients with an index>0), and 86.5% specificity (CI95%: 81.5%;91.5%) against other rheumatic diseases. Overall, the specificity against RA was 92.5% (9 false positives), 76.2% against systemic sclerosis patients (5 false positives), 66.7% against dermatomyositis (3 false positives), 62.5% against vasculitis (3 false positives), 75.0% against Sjogren's Syndrome (2 false positives), 71.4% against polymyositis (2 false positives patients). None of the patients with granulomatosis with polyangiitis (2 patients), fibromyalgia (2 patients) and Sjogren Syndrome with fibromyalgia (one patient) were positive (100% specificity). Overall specificity against healthy subjects was 97.6% (200/205)(CI95%: 95.4%;99.7%). We also determined the performance characteristics when anti-dsDNA and ANA were combined. Sensitivity was 89.0% (187/210 positives) while specificity was only 58.4% against other diseases (104/178 negatives).

Effect of SLE disease activity on the CB-CAPs results and Index Score

A total of 41 SLE patients (19.6%) presented with active disease as defined by a SLEDAI≥6. As presented in **Table V**, patients presenting with active disease had elevated levels of ANA, EC4d, BC4d, PC4d and reduced levels of ECR1 ($p<0.004$). ROC analysis indicated that ANA above 90 units (AUC=0.694) was associated with a 3.9-fold (CI95%: 1.7-9.8) higher likelihood of active disease. Similarly, EC4d above 14.8 units (ROC AUC=0.646), BC4d above 71.5 units (ROC AUC=0.643) and PC4d above 6.3 units (ROC AUC=0.718) were associated with a 3.4-fold (CI95%: 1.6-7.4), a 4.3-fold (CI95%: 1.9-10.8) and 5.3-fold (CI95%: 2.3-13.5) greater likelihood of active disease, respectively. Alternatively, ECR1 below 10 net MFI (AUC=0.694) were associated with a 4.2-fold (CI 95%: 1.9-9.3) higher likelihood of active disease.

Moreover, the index score calculated to differentiate SLE from other diseases was significantly higher in SLE patients having active disease compared to those with non active disease (**Table V**). Among anti-dsDNA negative patients with active disease (14/41), 92.9% (13/14) presented a positive index score whereas among anti-dsDNA negative patients with non-active disease (133/168), 69.2% (92/133) of them presented a positive index score. Finally, a positive index was observed in 64.8% of patients having a SELENA-SLEDAI score below 2 points (46/71).

DISCUSSION

The primary objective of this study was to evaluate the contribution of CB-CAPs to SLE diagnosis in a large multi-centered cross-sectional study. Our data confirm that excessive C4d complement deposition on erythrocytes, B cells, platelets and alternatively CR1 expressed on erythrocytes are generally several-fold different in SLE compared to other diseases (8-10). These data are consistent with the notion that complement activation through classical pathway together with decreased CR1 mediated clearance of immune complexes are implicated in the pathogenesis of SLE.

However, significant overlap in the expression of these markers was observed between SLE and other rheumatic diseases thereby indicating that none of the individual CB-CAPs achieved adequate balanced clinical sensitivity and specificity alone (21). Anti-dsDNA was an insensitive (29.5%) yet specific marker (>95%), whereas ANA was a sensitive but poorly specific marker. We relied on the high anti-dsDNA antibody specificity (low false positive rate) to evaluate the incremental diagnostic value of CB-CAPs ANA and anti-MCV. Among anti-dsDNA negative individuals, the stepwise addition of log normalized EC4d and BC4d markers significantly increased sensitivity (**Table III**) while maintaining adequate specificity. The specificity gain further contributed by anti-MCV was dependent on RA (specificity against RA was 87.7%

without vs. 97.4% with anti-MCV) and enabled the selection of a more optimal cutoff increasing overall SLE sensitivity. The index score (>0) combining the weighed sum of ANA, EC4d, BC4d together with anti-MCV was sensitive (71.6%) and specific (90.1%) for SLE. Altogether, the combination of anti-DNA and the index score improved the clinical sensitivity vs. anti-DNA alone from 29.5% to 80.0%. This 50.5% improvement in clinical sensitivity largely outweighed the 9.6% loss in specificity from 96.1% to 86.5%. Moreover, when compared to anti-dsDNA and ANA combined, anti-dsDNA with the index resulted into a 30.3% improvement in specificity (59.0% vs. 89.3%) with only a 10.0% loss in sensitivity (89.0% vs. 79.0%). Our data clearly demonstrate the value of combining serological and CB-CAPs markers to achieve adequate balanced sensitivity and specificity for the diagnosis of SLE.

A secondary objective was also to evaluate the effect of disease activity as assessed using the SEDAI on the performance of these diagnostic biological markers. Our preliminary data confirm that elevated EC4d, BC4d and PC4d and reduced ECR1 are associated with active disease as previously suggested (12;13). However, our data were only cross-sectional and prospective longitudinal studies will be essential to establish the true value of CB-CAPs measurements for monitoring SLE disease. Our analysis also revealed that the index score yielded lower clinical sensitivity among anti-dsDNA negative patients presenting with non-active disease (69.2%) compared to those presenting with active disease (92.9%). However, among patients with a SELENA- SLEDAI below two points the index score yielded a sensitivity of 64.8%. Altogether these data illustrate the capability of the index score to classify SLE among anti-dsDNA negative patients with mild symptoms.

In conclusion, our data establish the value of CB-CAPs for the differential diagnosis of SLE. An assay panel combining anti-dsDNA, ANA, anti-MCV antibodies together with cell surface EC4d and BC4d is sensitive and specific for SLE.

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FIGURE LEGEND

Figure 1: Index score in the differential diagnosis of SLE

Panel A: Index score values among 523 anti-dsDNA negative individuals corresponding to 204 normal healthy volunteers (NHV), 171 other disease and 148 SLE patients. The index score was calculated as the weighed sum of ANA positivity log normalized EC4d and BC4d net MFI and anti-MCV negativity. Estimates are provided in Table IV. For example a patient with positive ANA (≥20 units), negative anti-MCV (<70 units), EC4d level of 37 Net MFI and BC4d level of 102 Net MFI presents an index score of 3.53 [=-8.080 + 2.2833x1 - 2.6575x0 + 1.1526xlog(37) + 1.1165xlog(102)]

Panel B: Sensitivity and specificity (for other diseases) of the index score among anti-dsDNA negative individuals. Symbols correspond to the actual performances. Dotted lines correspond to 95% confidence intervals (20). Dark symbols correspond to sensitivity; Open symbols correspond to specificity.

TABLES

Table I: Characteristics of 210 SLE patients enrolled in the study.

Criteria were collected based on history for each patient.

Gender (females), N (%)	190 (90%)
Age, median (range)	41 (19-81)
Ethnicity, N (%)	
Caucasians	75 (36%)
African Americans	76 (36%)
Asians	16 (8%)
Hispanics	40 (19%)
Others	3 (1%)
Malar rash, N (%)	91 (43%)
Discoid rash, N (%)	29 (14%)
Photosensitivity, N (%)	76 (36%)
Oral ulcers, N (%)	59 (28%)
Arthritis, N (%)	154 (53%)
Serositis, N (%)	59 (28%)
Pleuritis	40 (19%)
Pericarditis	26 (12%)
Renal disorder, N (%)	86 (41%)
Proteinuria >0.5 g/d	80 (38%)
3+ cellular casts	9 (4%)
Neurologic disorder, N (%)	15 (7%)
Seizures	14 (7%)
Psychosis without other causes	2 (1%)
Hematologic disorder, N (%)	113 (54%)
Hemolytic anemia	8 (4%)
Leukopenia (<4000/L)	59 (28%)
Lymphopenia (<1500/L)	53 (25%)
Thrombocytopenia (<100,000/L)	29 (14%)
Immunologic disorder, N (%)	171 (81%)
anti-dsDNA	140 (67%)
anti-Sm	47 (22%)
anti-phospholipid	57 (27%)
Antinuclear antibodies, N (%)	205 (98%)
SELENA-SLEDAI*	
median (range)	2 (0-22)
≥6	41 (19.6%)

* SLEDAI was available in 209 SLE patients and measured within 30 days of blood draw.

Table II: **Diagnostic assay results among SLE, other diseases and healthy subjects**. (Net MFI: Net Mean Fluorescence intensity). Percent false positive (%; 1-specificity) is indicated for other diseases and normal healthy volunteers. Results are expressed as average±SEM.

	Normal Healthy N=205	Other diseases N=178	SLE N=210
ANA			
units/L	13±1	40±4	88±3
units/L≥20[a]	9.3%	41.0%	89.0%
Anti-dsDNA			
units/L	40±4	61±8	228±17
units/L> 301[a]	0.5%	3.9%	29.5%
Anti-MCV			
units/L	2±0.6	160±21	9±2
units/L> 70[b]	0.5%	36.0%	1.9%
EC4d			
Net MFI	5.3±0.4	6.3±0.3	17.6±1.2
Net MFI>9[c]	7.3%	16.9%	70.0%
BC4d			
Net MFI	23.5±1.1	34.9±3.4	110.4 ±7.2
Net MFI>48[c]	4.4%	13.5%	65.7%
PC4d			
Net MFI	2.0±0.4	3.6±0.3	16.3±2.2
Net MFI >7[c]	0.5%	7.3%	46.2%
ECR1			
Net MFI	20.7±0.5	16.1±0.5	13.3±0.4
Net MFI<16[c]	30.2%	51.1%	73.8%

[a] manufacturer recommended cutoffs; [b]optimal cutoff with high specificity vs. RA. [c]cutoffs established by ROC analysis (18).

Table III: Stepwise addition of ANA, EC4d, BC4d and anti-MCV improves diagnostic performances among anti-dsDNA negative patients

For each model, an index score corresponding to a weighted sum of the markers (as appropriate) was calculated. Clinical sensitivities specificities with ROC AUC were calculated for an index score>0.

	ANA≥20	ANA≥20 + log(EC4d)	ANA≥20 + log(EC4d) + log(BC4d)	ANA≥20 + log(EC4d) + log(BC4d) + Anti-MCV>70
Sensitivity SLE	84.5% (125/148)	65.5% (97/148)	68.2% (101/148)	71.6% (106/148)
Specificity Other diseases	60.8% (104/171)	80.1% (137/171)	86.0% (147/171)	90.1% (154/171)
Specificity Normal healthy	90.7% (185/204)	97.5% (199/204)	99.0% (202/204)	98.0% (200/204)
ROC AUC	0.808±0.0185	0.887±0.0165	0.903±0.0159	0.918±0.0146

Table IV: Multivariate logistic regression analysis among anti-dsDNA negative patients. ECR1 and PCd4 did not contribute significantly (p=0.09 and p=0.34, respectively). Log corresponds to the natural log of net MFI for EC4d and BC4d. for each patient the index score corresponded to the weighed sum of each of the component including intercept.

	Estimate (SEM)	Odd Ratio (CI 95%)	P value
(Intercept)	-8.08±0.84		$< 2x10^{-16}$
ANA≥20 Units	2.28±0.30	9.81 (5.46-17.62)	$2.17x10^{-14}$
AntiMCV>70 Units	-2.66±0.64	0.07 (0.02-0.25)	$3.40x10^{-05}$
LOG(EC4d)	1.15±0.28	3.17 (1.85-5.43)	$2.85x10^{-05}$
LOG(BC4d)	1.12±0.24	3.05 (1.92-4.86)	$2.42x10^{-06}$

Table V: **CB-CAPs levels in SLE patients with non-active vs. active disease**

A SLEDAI score≥6 differentiated active from non active disease. Results are expressed as median interquartile range.

	Non-active disease N=168	Active disease N=41	P value
ANA			
units/L	87 (33-131)	126 (98-140)	<0.001
units/L≥90	47.6%	78.0%	<0.001
EC4d			
Net MFI	11.4 (7.4-19.5)	16.6 (11.3-26.0)	<0.004
Net MFI>14.8	33.9%	63.4%	<0.001
BC4d			
Net MFI	66.7 (35.1-130.0)	117.0 (75.2-188.6)	<0.005
Net MFI>71.5	45.2%	78.0%	<0.001
PC4d			
Net MFI	5.2 (2.4-10.8)	13.9 (7.3-43.4)	<0.001
Net MFI >6.3	39.9%	78.0%	<0.001
ECR1			
Net MFI	13.5 (9.2-17.9)	9.3 (6.7-12.4)	<0.001
Net MFI>10.2	67.9%	34.1%	<0.001
INDEX			
Index	1.75 (-0.02-2.92)	2.85 (1.81-3.63)	<0.001
Index≥0	74.4%	97.6%	<0.001

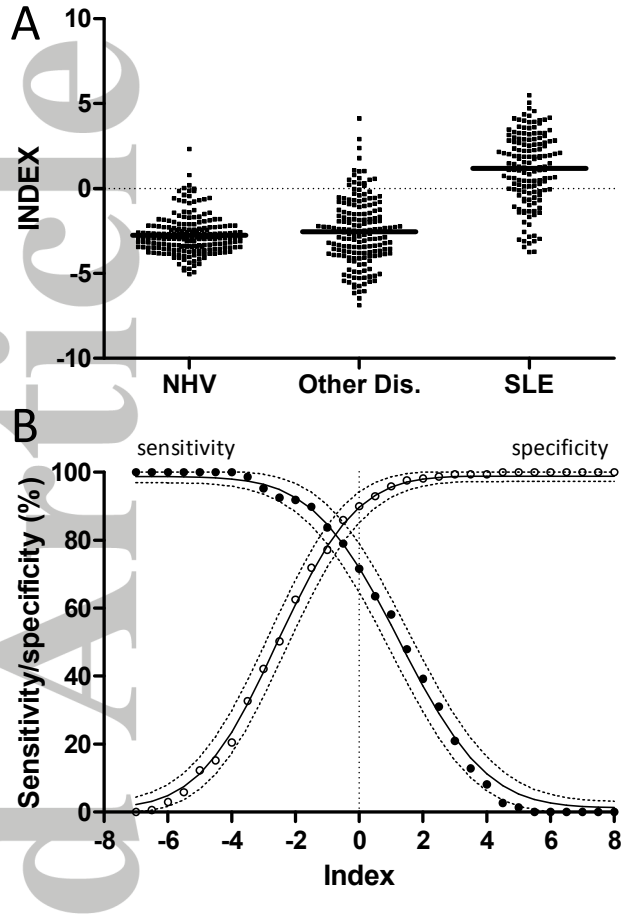

Figure 1: Index score in the differential diagnosis of SLE
Panel A: Index score values among 523 anti-dsDNA negative individuals corresponding to 204 normal healthy volunteers (NHV), 171 other disease and 148 SLE patients. The index score was calculated as the weighed sum of ANA positivity log normalized EC4d and BC4d net MFI and anti-MCV negativity. Estimates are provided in Table IV. For example a patient with positive ANA (≥20 units), negative anti-MCV (<70 units), EC4d level of 37 Net MFI and BC4d level of 102 Net MFI presents an index score of 3.53 [=-8.080 + 2.2833x1 - 2.6575x0 + 1.1526xlog(37) + 1.1165xlog(102)] *Panel B*: Sensitivity and specificity (for other diseases) of the index score among anti-dsDNA negative individuals. Symbols correspond to the actual performances. Dotted lines correspond to 95% confidence intervals (20). Dark symbols correspond to sensitivity; Open symbols correspond to specificity.