Four Anti-dsDNA Antibody Assays in Relation to Systemic Lupus Erythematosus Disease Specificity and Activity

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ABSTRACT. *Objective*. Analysis of antibodies against dsDNA is an important diagnostic tool for systemic lupus erythematosus (SLE), and changes in anti-dsDNA antibody levels are also used to assess disease activity. Herein, 4 assays were compared with regard to SLE specificity, sensitivity, and association with disease activity variables.

Methods. Cross-sectional sera from 178 patients with SLE, of which 11 were followed consecutively, from a regional Swedish SLE register were analyzed for immunoglobulin G (IgG) anti-dsDNA by bead-based multiplex assay (FIDIS; Theradig), fluoroenzyme-immunoassay (EliA; Phadia/Thermo Fisher Scientific), *Crithidia luciliae* immunofluorescence test (CLIFT; ImmunoConcepts), and line blot (EUROLINE; Euroimmun). All patients with SLE fulfilled the 1982 American College of Rheumatology and/or the 2012 Systemic Lupus International Collaborating Clinics (SLICC-12) classification criteria. Healthy individuals (n = 100), patients with rheumatoid arthritis (n = 95), and patients with primary Sjögren syndrome (n = 54) served as controls.

Results. CLIFT had the highest SLE specificity (98%) whereas EliA had the highest sensitivity (35%). When cutoff levels for FIDIS, EliA, and EUROLINE were adjusted according to SLICC-12 (i.e., double the reference limit when using ELISA), the specificity and sensitivity of FIDIS was comparable to CLIFT. FIDIS and CLIFT also showed the highest concordance (84%). FIDIS performed best regarding association with disease activity in cross-sectional and consecutive samples. Fisher's exact test revealed striking differences between methods regarding associations with certain disease phenotypes.

Conclusion. CLIFT remains a good choice for diagnostic purposes, but FIDIS performs equally well when the cutoff is adjusted according to SLICC-12. Based on results from cross-sectional and consecutive analyses, FIDIS can also be recommended to monitor disease activity. (First Release Feb 15 2015; J Rheumatol 2015;42:817–25; doi:10.3899/jrheum.140677)

Key Indexing Terms:
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AUTOANTIBODIES   INFLAMMATION   RHEUMATIC DISEASE

Systemic lupus erythematosus (SLE) is a heterogeneous disease characterized by multiorgan involvement and circulating autoantibodies against a variety of antigens, most notably nuclear antigens, i.e., antinuclear antibodies (ANA)[1]. A positive ANA test by immunofluorescence microscopy remains a hallmark in SLE, although it is not mandatory according to the American College of Rheumatology classification criteria from 1982 (ACR-82)[2] or to the recently postulated Systemic Lupus International Collaborating Clinics (SLICC) classification criteria for SLE[3].

ANA targeting DNA were demonstrated by several independent research groups already in 1957[4]. In 1966, 1 of these groups demonstrated circulating antibodies against native/dsDNA (anti-dsDNA) as well as circulating DNA in patients with SLE, suggesting a pathogenic connection[5].

This concept was further implicated by elution of DNA and anti-DNA antibodies from glomeruli of patients with SLE nephritis[6,7,8]. The advantage of measuring antibodies against strictly dsDNA (in the form of circular mitochondrial DNA in kinetoplasts of *Crithidia luciliae*) was brought up by Aarden, *et al*, who developed the *C. luciliae* immuno-fluorescence test (CLIFT)[9]. Immunoglobulin G (IgG) anti-dsDNA analyzed by CLIFT is considered fairly specific for SLE[4,10], but it is also typical of autoimmune hepatitis type 1 and can be induced in patients treated with sulfasa-lazine or tumor necrosis factor inhibitors[11,12]. Anti-dsDNA antibodies may be present before onset of clinical disease and are often associated with severe manifestations, such as glomerulonephritis[13,14]. As measured by CLIFT, 40–80% of patients with SLE have been reported to be anti-dsDNA–posi-tive over time, depending on the disease activity and severity[4,10,15]. SLE disease flares are frequently associated with increasing serum levels of anti-dsDNA concomitantly with decreased levels of complement proteins C1q, C3, and C4, especially in SLE nephritis[16].

ELISA for the measurement of anti-dsDNA have repeatedly been found to have lower disease specificity compared to CLIFT[16,17,18,19,20]. The new 2012 SLICC (SLICC-12) criteria[3] have implemented this knowledge and recommend a cutoff for ELISA that is double the "laboratory reference"[3].

Many laboratories in Scandinavia (including our own) use CLIFT not only for diagnostic purposes, but also when monitoring changes in disease activity over time. Owing to the relatively time-consuming CLIFT procedure, as well as because of its semiquantitative nature, another rapid test with improved ability to reflect clinically relevant fluctua-tions in anti-dsDNA levels is highly warranted given a specificity comparable with CLIFT. Our present study was undertaken to evaluate alternative assays that could complement or replace the CLIFT. Four different anti-dsDNA detection assays were thus compared regarding disease specificity, disease sensitivity, and disease activity: the CLIFT, a fluoroenzyme-immunoassay (EliA), a bead-based multiplex assay (fluorescent microsphere immunodetection system; FIDIS), and a line blot assay (EUROLINE). In view of the new SLICC-12 criteria, diagnostic specificity and sensitivity performances of the different assays were evaluated with and without an elevated cutoff limit. Cross-sectional, as well as longitu-dinal, serum samples from a well-characterized Swedish SLE cohort formed the basis of this investigation.

MATERIALS AND METHODS
Patients and controls. A total of 178 patients meeting the ACR-82 and/or the SLICC-12 were included in the study[2,3]. All patients with SLE took part in a prospective, structured followup program at the rheumatology clinic, Linköping University Hospital, Sweden[21,22]. A total of 155 patients fulfilled the ACR-82 (87%), 173 fulfilled the SLICC-12 (97%), and 150 patients fulfilled both criteria (84%). Prevalent (91%) as well as incident

cases (9%) were recruited consecutively during 2008–2011, and the disease duration at study inclusion ranged from 0 to 45 years with a mean of 11 years. Disease activity was recorded at every visit using the SLE Disease Activity Index 2000 (SLEDAI-2K)[23] as well as the physician's global assessment of disease activity (PGA 0–4)[24]. The mean SLEDAI-2K was 2.3 (range 0–16), mean age at inclusion was 50 years (range 18–88), 90% were women, and 90% were white. The mean number of fulfilled ACR-82 criteria by patients was 4.8, and 21% fulfilled the ACR-82 renal disorder criterion (other criteria are presented in Table 1). Hydroxychloroquine (HCQ) alone was prescribed to 42% of the patients, 30% were prescribed other disease-modifying antirheumatic drugs (DMARD) with or without HCQ, and 65% were treated with prednisolone. The most frequently prescribed DMARD were mycophenolate mofetil (11% of the patients) and methotrexate (MTX; 10%) whereas other DMARD (azathioprine, sirolimus, rituximab, and cyclosporine) were less common. About half of the patients (48%) had tested anti-dsDNA–positive by CLIFT (the clinical routine method at Linköping University Hospital) at any occasion during their disease course. Eleven patients with SLE representing different disease manifestations and with varying disease activity (SLEDAI-2K difference of ≥ 6) over time were selected for consecutive anti-dsDNA analyses.

Patients with primary Sjögren syndrome (pSS) and patients with rheumatoid arthritis (RA) served as disease controls. Serum samples were collected from 54 patients (96% women; mean age 62 yrs) meeting the American-European consensus criteria for pSS[25]. Half of the patients with pSS (50%) had a history of extraglandular disease. Of the patients, 89% were positive for anti-SSA (with or without anti-SSB), 50% received prednisolone, 54% were treated with HCQ, and 29% were prescribed other DMARD of which MTX was the most common (15%).

The patients with RA (n = 95) were from a Swedish RA cohort called TIRA (Swedish acronym for "early interventions in RA"). The patients were newly diagnosed (≤ 12 mos since the first joint swelling) and included in the TIRA cohort between 1996 and 1998[26]. None of the patients were receiving DMARD at inclusion in the study. The mean age was 55 years, 69% were women, 64% were anticyclic citrullinated peptide 2 (anti-CCP2)-positive, and 60% were rheumatoid factor (RF)-positive. During 8 years of followup, none of the patients developed SLE. Control sera from 100 blood donors (50% women; mean age 41 yrs) at Linköping University Hospital were also analyzed.

IF-microscopy: CLIFT. Microscope slides with fixed *Crithidia luciliae* (ImmunoConcepts) were incubated for 30 min with serum diluted 1:10 in phosphate buffered saline (pH 7.4). After washing and a 30-min incubation with fluorescein-isothiocyanate conjugated γ-chain–specific polyclonal rabbit anti-human IgG (DAKO A/S), the slides were mounted in Pro-Long Gold fluorescent mounting medium (Molecular Probes-Life technologies, Thermofisher Scientific) and evaluated by indirect IF-microscopy at 400× magnification. CLIFT-positive sera at the screening dilution (> 99th percentile among 100 blood donors, 50 women/50 men) were endpoint titrated in 2-fold dilution steps.

Line blot: EUROLINE. Anti-dsDNA measurement by line blot was performed with the ANA Profile 5 EUROLINE test kit on a EURO-Blotmaster instrument (Euroimmun AG). The assay, which uses native dsDNA isolated from salmon testes as antigen, was run according to manufacturer's instructions. Briefly, the immunoblot strips were incubated with a diluted serum sample (1:101) for 30 min. After washing, alkaline phosphatase-labeled γ-chain–specific polyclonal goat anti-human IgG was added and incubated for 30 min. The strips were then washed, and thereafter incubated in substrate solution (nitrobluetetrazoliumchlorid/5-bromo-4-chloro-3-indolylphosphate) for 10 min. The reaction was stopped by washing the strips with distilled water and the test strips were evaluated with EUROLINEScan (Euroimmun AG). The blot intensity was quantified by densitometry. A signal intensity of ≥ 11 was considered positive according to the manufacturer, whereas ≥ 22 was the cutoff used when applying the strict SLICC-12 criteria.

Fluoroenzyme-immunoassay: EliA. Anti-dsDNA detection by EliA uses

Table 1. Association of SLE disease criteria and anti-dsDNA assessed by 4 different assays. The analysis (Fisher's exact test) is not adjusted for multiple comparisons.

Cumulative ACR-82 Criteria* (% fulfilling the criterion)	FIDIS p	FIDIS OR (95% CI)	EliA p	EliA OR (95% CI)	CLIFT p	CLIFT OR (95% CI)	EUROLINE p	EUROLINE OR (95% CI)
1. Malar rash (45%)	0.325	1.41 (0.74–2.69)	0.753	1.12 (0.60–2.08)	0.726	0.84 (0.42–1.70)	0.870	1.08 (0.57–2.05)
2. Discoid rash (17%)	0.084	0.40 (0.14–1.10)	0.302	0.60 (0.25–1.43)	0.356	0.55 (0.20–1.54)	0.391	0.62 (0.25–1.54)
3. Photosensitivity (56%)	**0.047**	0.49 (0.26–0.95)	0.752	0.86 (0.46–1.60)	**0.003**	0.33 (0.16–0.67)	0.104	0.58 (0.31–1.11)
4. Oral ulcers (10%)	0.587	1.32 (0.46–3.79)	**0.035**	3.00 (1.08–8.31)	0.249	1.83 (0.63–5.28)	0.405	1.70 (0.61–4.73)
5. Arthritis (78%)	0.692	0.81 (0.38–1.73)	0.447	0.71 (0.34–1.47)	0.674	1.30 (0.55–3.10)	1.0	0.97 (0.45–2.11)
6. Serositis (38%)	0.315	1.42 (0.73–2.72)	0.746	0.87 (0.46–1.65)	0.588	1.26 (0.63–2.55)	0.737	0.86 (0.44–1.67)
7. Renal disorder (21%)	**0.01**	2.70 (1.29–5.68)	**0.004**	2.98 (1.43–6.23)	0.054	2.23 (1.03–4.85)	0.171	1.69 (0.80–3.57)
8. Neurologic disorder (6%)	1.0	0.98 (0.25–3.98)	0.321	1.95 (0.54–7.00)	1.0	0.77 (0.15–3.79)	0.174	0.41 (0.11–1.49)
9. Hematologic disorder (57%)	**0.013**	2.42 (1.21–4.83)	0.525	1.28 (0.69–2.41)	0.480	1.35 (0.67–2.73)	0.621	1.23 (0.65–2.35)
1, 2, or 3. Skin (75%)	0.086	0.51 (0.25–1.04)	1.0	1.04 (0.51–2.14)	**0.001**	0.29 (0.14–0.60)	0.091	0.53 (0.26–1.08)

Significant data are in bold face. * Immunologic disorder (ACR criterion 10, 49% of patients) and antinuclear antibodies (ACR criterion 11, 98% of patients) are not reported because all anti-dsDNA–positive patients by CLIFT fulfill these criteria. Manufacturer's cutoff (FIDIS, EliA, and EUROLINE) or laboratory reference cutoff (CLIFT) for positivity were used. SLE: systemic lupus erythematosus; ACR-82: American College of Rheumatology 1982 criteria; FIDIS: bead-based multiplex assay; EliA: fluoroenzyme-immunoassay; CLIFT: *Crithidia luciliae* immunofluorescence test; EUROLINE: line blot.

recombinant circular plasmid dsDNA as antigen and was performed on the Phadia250 instrument (EliA dsDNA; Phadia, now part of Thermo Fisher Scientific). The assay was run according to the manufacturer's instructions. In short, serum samples were added to the instrument where they were diluted 1:10 and added to antigen-coated wells. After incubation and washing, monoclonal γ-chain–specific anti-human IgG conjugated with β-galactosidase was added to the wells. Development solution (0.01% 4-methylumbelliferyl-β-D-galactoside) was then applied and the reaction was terminated by adding a stop solution (4% sodium carbonate). An antibody concentration of ≥ 16 IU/ml (manufacturer's recommendation) or ≥ 32 IU/ml (SLICC-12 recommendation) was considered positive. Samples above the assay range (≥ 379 IU/ml) were given a value of 450 IU/ml.

Bead-based multiplex assay with Luminex's xMAP technology: FIDIS. This bead assay (Theradiag) uses recombinant circular plasmid dsDNA as antigen. The FIDIS Connective profile test was run according to the manufacturer's instruction. Briefly, 100 μl of samples diluted 1:201 were added to beads coated with 13 different ANA-associated antigens for 30 min in a 96-well plate with filter membrane bottoms. After washing with a vacuum device, a phycoerythrin-conjugated γ-chain–specific detection antibody was added for 30 min. After further washing, the plate was read and evaluated using a FIDIS analyzer and the MLX-Booster software, using Luminex's xMAP technology. A concentration of ≥ 40 IU/ml was considered positive according to the manufacturer and ≥ 80 IU/ml was the cutoff when applying the SLICC-12 criteria.

Other laboratory analyses. Visits for all patients with SLE included analysis of blood cell counts, urine erythrocytes, urine albumin, erythrocyte sedimentation rate (ESR), interferon-α (IFN-α), levels of complement proteins C3 and C4 measured by nephelometry in fresh frozen plasma samples, and classic complement function assessed by a hemolytic assay. IFN-α was measured at Uppsala University by a dissociation-enhanced lanthanide fluorescent immunoassay[27,28]. Complement analyses were performed at Uppsala University Hospital or Linköping University Hospital. In sera from patients with RA, anti-CCP2 was detected by ELISA and agglutinating RF was assessed by nephelometry at Linköping University Hospital.

Ethics. Oral and written informed consent was obtained from all patients and controls. Study protocols regarding patients with SLE and patients with RA were approved by the Linköping University Ethical Review Board (No. M75-08 and 96035, respectively), and the study protocol for patients with pSS was approved by the Uppsala University Ethical Review Board (No. 2006/217/2).

Statistics. Concordance between methods was defined as the sum of double-positive samples and double-negative samples, divided by the total number of samples, multiplied by 100. Because values obtained by CLIFT are ordinal and EUROLINE is considered qualitative according to the manufacturer, continuous variables were tested with nonparametric tests (Spearman correlation, Mann-Whitney U test, and Wilcoxon signed-rank test) to accomplish comparability among all anti-dsDNA measurement methods. Fisher's exact test was used for categorical data. Receiver-operator characteristics (ROC) were used to calculate cutoff limits for a given specificity. Two-tailed p values of < 0.05 were considered significant. Statistical analyses were performed with SPSS Statistics 21 (IBM) or GraphPad Prism 5, version 5.04 (GraphPad Software).

RESULTS

SLE specificity. Serum anti-dsDNA antibodies were determined in patients and healthy controls, and the specificity and sensitivity for SLE was calculated for each of the 4 methods using the cutoff according to the manufacturer (FIDIS, EliA, and EUROLINE) or laboratory reference (CLIFT; Figure 1). The highest percentage of anti-dsDNA–positive patients with SLE (i.e., sensitivity) was found using EliA (35%), whereas CLIFT had the lowest sensitivity (24%). In contrast, CLIFT displayed the highest disease specificity for SLE (98%). FIDIS had the lowest SLE specificity, but in contrast to EliA and EUROLINE, it did not identify any healthy individuals. Increasing the cutoff for EUROLINE, FIDIS, and EliA to double manufacturer's reference (according to recommendations in the SLICC-12 criteria for the use of ELISA; Figures 1B–D) resulted in excellent specificity for all methods (98–99%), but also reduced the sensitivity below the sensitivity for CLIFT. A ROC curve analysis rendered the best area under the curve for EliA (0.712), followed by EUROLINE (0.621), CLIFT (0.614), and FIDIS (0.571), but adjusting the cutoff limits for FIDIS, EliA, and EUROLINE to achieve an acceptable specificity (i.e., comparable to CLIFT; 98.4%) rendered the highest sensitivity for FIDIS (24%, cutoff at 77 IU/ml) followed by EliA (19%, cutoff at 38 IU/ml) and EUROLINE (19%, cutoff at a signal intensity of 19).
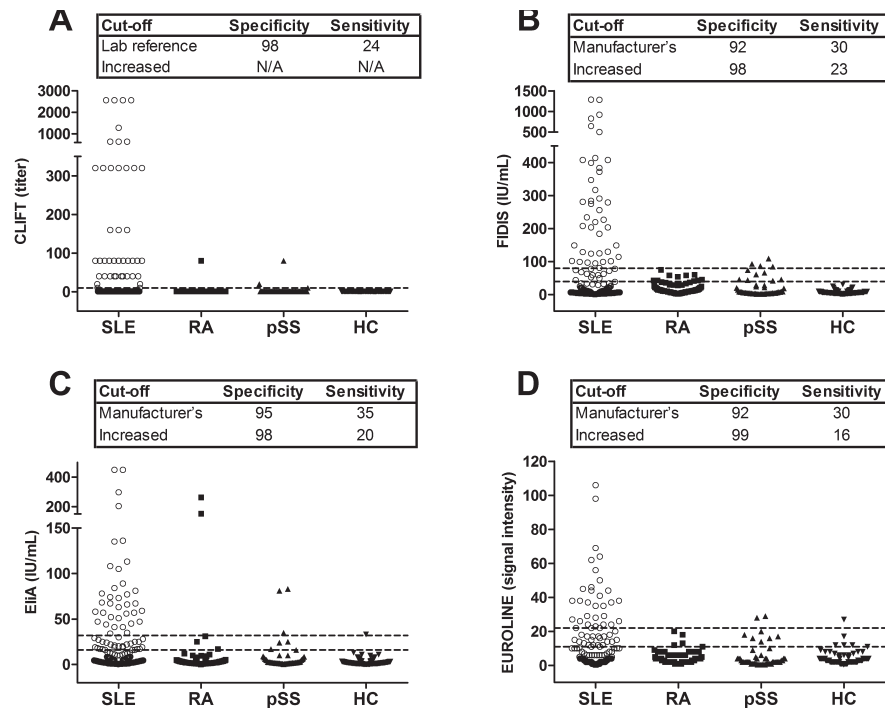
Figure 1. Anti-dsDNA in patients with SLE and control groups as measured by CLIFT (A), FIDIS (B), EliA (C), and EUROLINE (D). SLE disease specificity of different anti-dsDNA antibody assays was calculated in relation to control groups (RA, pSS, and HC). The dashed lines indicate cutoff for positivity according to laboratory reference (A), manufacturer's recommendation (lower line in panel B–D), or double manufacturers' references (upper line in panel B–D). Specificity and sensitivity for the respective cutoff limits are denoted in the tables above the graphs. SLE: systemic lupus erythematosus; CLIFT: *Crithidia luciliae* immunofluorescence test; FIDIS: bead-based multiplex assay; EliA: fluoroenzyme-immunoassay; EUROLINE: line blot; RA: rheumatoid arthritis; pSS: primary Sjögren syndrome; HC: healthy controls; N/A: not applicable.

Concurrence between methods. Correlations and concordances between methods were evaluated in the 178 cross-sectional SLE sera. Samples below the cutoff were given a value of 0 to avoid influence of level differences among double-negative samples in the correlation analyses. All methods correlated significantly with each other and had a concordance of ≥ 72% (Figure 2). The strongest correlation and concordance at manufacturers' cutoffs was found between CLIFT and FIDIS (Figure 2A; rho = 0.623, p < 0.0001, concordance = 84%), whereas FIDIS and EliA had the highest correlation and concordance at the increased cutoff (numbers in parenthesis in Figure 2B; rho = 0.641, p < 0.0001, concordance = 87%). EUROLINE versus EliA, and EUROLINE versus FIDIS, displayed the weakest correlations as well as the lowest concordances (Figures 2E and 2F). When applying increased cutoff levels for FIDIS, EliA, and EUROLINE, all concordances increased (concordances in parentheses in Figures 2A–F) except the one between EliA and FIDIS, which remained at 84% (Figure 2A) and between EUROLINE and CLIFT, which was lowered (Figure 2C).

The overlap between methods (positive by ≥ 2 methods at manufacturer's cutoff) in the disease control groups was 32% for pSS and 11% for RA, with the highest overlap between CLIFT and FIDIS. Anti-dsDNA–positive patients with pSS (measured by any method) did not differ significantly from anti-dsDNA–negative patients with pSS with regard to the presence of 1 or more extraglandular manifestations. Patients with RA judged anti-dsDNA–positive with FIDIS (manufacturer's cutoff) were also more often positive for RF (9 out of 10 patients, p = 0.047) and anti-CCP2 (10/10, p = 0.013) than those testing anti-dsDNA–negative with FIDIS. In contrast, only 2 of 5 anti-dsDNA–positive patients with RA assessed by EliA and EUROLINE were tested RF-positive (p = 0.39). Further, patients with RA who were anti-dsDNA–positive by EliA (manufacturer's cutoff) tended to be anti-CCP–negative (4/5, p = 0.054).

Association of anti-dsDNA with disease phenotype. To evaluate whether any of the 4 anti-dsDNA assays identified specific disease phenotypes, we compared their association with individual and grouped ACR-82 criteria (Table 1). Patients meeting any skin criteria (ACR criteria 1 and/or 2 and/or 3) were less likely to be anti-dsDNA–positive by CLIFT. EliA was the only assay where anti-dsDNA was associated with oral ulcers, whereas FIDIS was the only method where anti-dsDNA was related to hematological
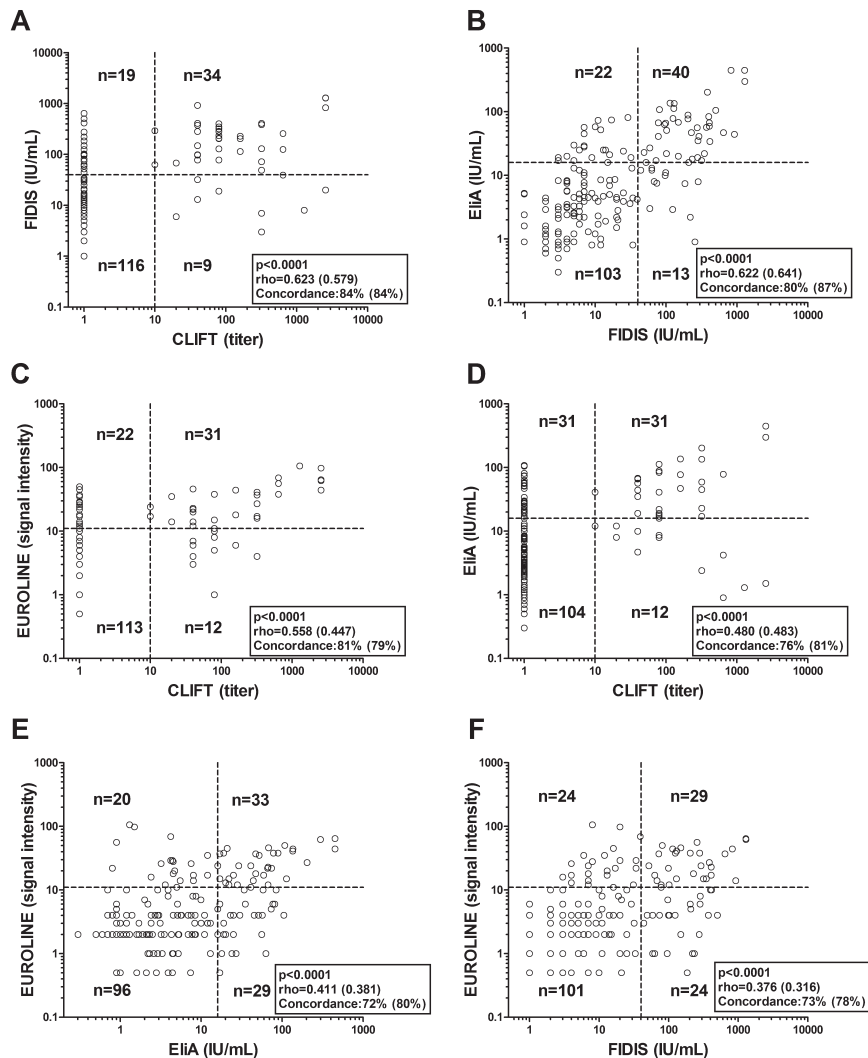
Figure 2. Correlation and concordance of anti-dsDNA assays in cross-sectional SLE samples. Panels (A–F) are sorted according to correlation. Certain dots represent more than 1 patient. Zero values are replaced with 1 for CLIFT and 0.5 for EUROLINE, to be contained in the log-scale of the axes. Dashed lines indicate the manufacturer's cutoff for positivity (EliA, FIDIS, and EUROLINE) or laboratory reference (CLIFT), and numbers designate the sum of patients in the specific quadrant. Values of p and rho are from Spearman correlation. Values below cutoff were given a value of 0 to avoid unwanted influence of double-negative samples in the correlation analyses. The rho values and assay concordances when adopting a cutoff limit double the manufacturer's recommendation are given in parentheses. Concordance is the sum of double-positive samples and double-negative samples, divided by the total number of patients (n = 178). SLE: systemic lupus erythematosus; CLIFT: *Crithidia luciliae* immunofluorescence test; EUROLINE: line blot; EliA: fluoro-enzyme-immunoassay; FIDIS: bead-based multiplex assay.

disorder. Patients fulfilling ACR criterion 7 (renal disorder) were more likely to be anti-dsDNA–positive by EliA and FIDIS. The ACR-10 criterion (immunological disorder), which among other autoantibodies includes anti-dsDNA (herein defined by CLIFT), was associated with anti-dsDNA positivity in all assays (not shown). There were no statistically significant associations between the use of specific DMARD and anti-dsDNA positivity by any of the methods (not shown). Manufacturer's cutoff (EliA, FIDIS, and EUROLINE) and laboratory reference cutoff (CLIFT) were used in these analyses.

Correlation with disease variables (cross-sectional analysis). To evaluate the association of anti-dsDNA antibody levels with disease activity measures (SLEDAI-2K, PGA, classical complement function, ESR, and circulating levels of C3, C4, and IFN-α), we compared the 4 methods

in patients with SLE (Table 2). The strongest correlations were found between anti-dsDNA and complement proteins and classical complement function. Anti-dsDNA assessed by FIDIS versus classical complement function showed the highest Spearman rank correlation coefficient (rho = –0.552, p < 0.0005). Overall, FIDIS displayed a higher correlation coefficient to the activity variables (Table 2).

Individual variations in anti-dsDNA. To evaluate whether anti-dsDNA levels reflect disease activity over time at an individual level, we compared anti-dsDNA levels between the occasions with highest and lowest disease activity, respectively, in 11 patients that were followed consecutively. The number of patients with a > 25% increase in anti-dsDNA level (with the highest anti-dsDNA level above manufacturer's cutoff for positivity) were calculated for each method. FIDIS and EliA detected the increase in disease activity in 7 of the patients (only 5 patients for EliA when the increased cutoff was applied) while CLIFT identified 5 patients and EUROLINE detected raised disease activity in 2 of the patients. Of the 11 consecutively followed patients, 9 were anti-dsDNA–positive by any method and at any visit (manufacturers' cutoff). Graphs showing anti-dsDNA levels and disease activity over time for each of these 9 patients are shown in Figures 3A–I. According to the graphs, FIDIS was superior or equal to any other method in 6 patients (Figures 3A–F), whereas EliA performed better or similarly compared to any other method in 2 patients (Figures 3A and 3B) and CLIFT was superior or equal to any other method in 3 patients (Figures 3E-G). Disease flares of the patients represented by Figures 3H and 3I, respectively, were not mirrored by anti-dsDNA by any of the methods, according to our judgment. The visual interpretation was made based on how well the disease flare(s), as denoted by SLEDAI and/or PGA, was reflected by an increase in anti-dsDNA level. EUROLINE was not included in the graphs because of its poor performance of reflecting disease activity variables in cross-sectional samples.

DISCUSSION

Based on the results of our present study, there is a great variability among anti-dsDNA assays, and a stricter cutoff limit must be applied to achieve an acceptable SLE specificity of FIDIS, EliA, and EUROLINE. Further, we found that FIDIS is superior to the other methods in monitoring disease activity, but also has a specificity and sensitivity comparable to CLIFT when a strict cutoff limit is applied.

Most Swedish clinical immunology laboratories still use CLIFT for primary (or confirmatory) analysis of anti-dsDNA antibodies. At our laboratory, we routinely perform IgG-specific CLIFT in all IgG-ANA–positive serum samples, regardless of IF-staining pattern. The use of a γ-chain–specific secondary antibody avoids detection of low-avidity IgM-class antibodies without clinical interest[29]. High-affinity IgA and/or IgM anti-dsDNA, which may be clinically relevant[30,31], were not considered in our study.

The relatively low SLE sensitivity of all assays in this study is likely due to the relatively low mean disease activity and low percentage of renal involvement (ACR criterion 7) among the patients with SLE. One plausible explanation for the low prevalence of renal involvement is the ethnicity that is linked to the risk of nephritis with a lower frequency of renal involvement in white populations[32]. Further, the study cohort includes the majority of patients with SLE in the region, meaning that the whole spectrum of SLE is present, including uncomplicated cases. The lowest sensitivity among the compared assays was found for CLIFT (manufacturer's cutoff/laboratory reference). The fact that CLIFT, compared to other methods, repeatedly demonstrated a low sensitivity but high diagnostic specificity[20,33,34] was considered in the formation of the SLICC-12 classification criteria for SLE[3]. In these criteria, a cutoff limit double laboratory reference is required when ELISA (without further specification) is used for anti-dsDNA quantification. However, the SLICC-12 criteria do not state whether this also applies for other "non-classical" assays (i.e., other than Farr and CLIFT) and

Table 2. Spearman correlation between disease variables and anti-dsDNA antibody levels assessed by 4 assays. Variables are organized according to correlation coefficient.

Variables	FIDIS		EliA		CLIFT		EUROLINE	
	rho	p	rho	p	rho	p	rho	p
Classical complement function, n = 169	–0.552	< 0.0005	–0.426	< 0.0005	–0.333	< 0.0005	–0.195	0.011
C4, n = 177	–0.495	< 0.0005	–0.362	< 0.0005	–0.284	< 0.0005	–0.209	0.005
C3, n = 178	–0.371	< 0.0005	–0.251	0.001	–0.218	0.003	–0.154	0.040
IFN-α, n = 178	0.323	< 0.0005	0.269	< 0.0005	0.215	0.004	0.137	0.068
ESR, n = 178	0.193	0.010	0.176	0.019	0.112	0.135	0.081	0.280
SLEDAI-2K*, n = 178	0.148	0.048	0.121	0.109	0.096	0.203	0.034	0.657
PGA, n = 178	0.109	0.148	0.160	0.033	–0.001	0.984	0.012	0.876

* Item for anti-dsDNA (by CLIFT) excluded. FIDIS: bead-based multiplex assay; EliA: fluoroenzyme-immunoassay; CLIFT: *Crithidia luciliae* immunofluorescence test; EUROLINE: line blot; IFN-α: interferon-α; ESR: erythrocyte sedimentation rate; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index 2000; PGA: physician global assessment.
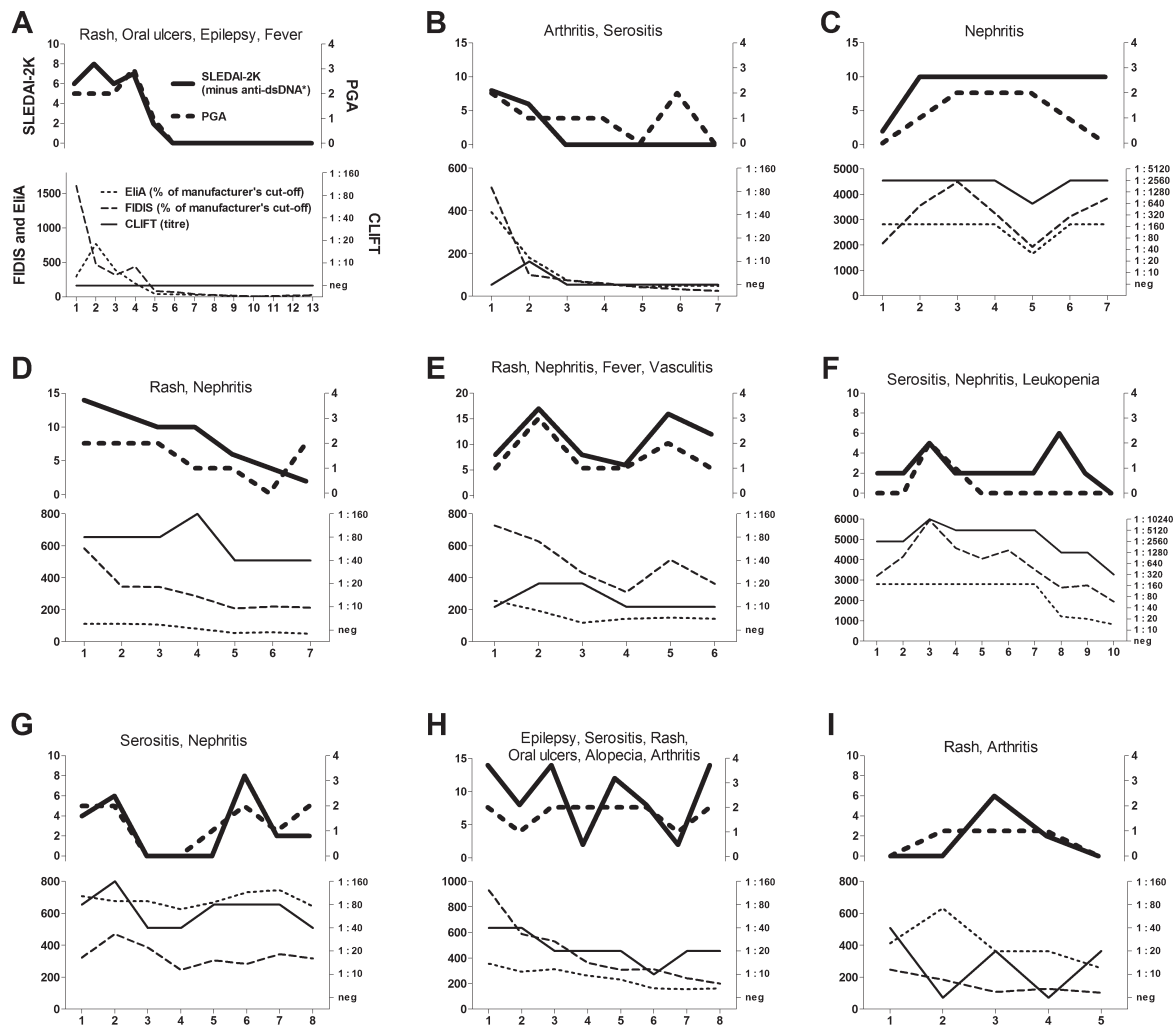
Figure 3. The graphs illustrate disease activity (PGA and SLEDAI-2K*), manifestations at flare, and anti-dsDNA levels in 9 patients (A–I) who were followed consecutively. Description of axes and lines are found in panel A. Axes scales are different among the graphs. Disease flare manifestations of patients with several flares were serositis and arthritis at first flare, and serositis at second flare (B); rash, nephritis, and fever at first flare, and rash, nephritis, and vasculitis at second flare (E); serositis and leukopenia at first flare, and nephritis at second flare (F); serositis and nephritis at first flare, and nephritis at second flare (G); epilepsy and serositis at first flare, and arthritis, rash, and epilepsy at second flare, and rash, alopecia, and epilepsy at third flare, and rash, alopecia, oral ulcers, and epilepsy at fourth flare (H). * Item for anti-dsDNA in SLEDAI was excluded. PGA: physician global assessment; SLEDAI-2K: Systemic Lupus Erythematosus Disease Activity Index 2000; EliA: fluoroenzyme-immunoassay; FIDIS: bead-based multiplex assay; CLIFT: *Crithidia luciliae* immunofluorescence test.

whether the laboratory reference denotes manufacturers' recommendations for cutoff, or to a cutoff determined by the clinical laboratory. In our study, an increased cutoff limit (double the manufacturer's recommendation) indeed increased the specificity to achieve comparability with CLIFT, but at the same time, the sensitivity became very low for EUROLINE; thus, also introducing a risk to overlook true anti-dsDNA–positive patients. The importance of a proper threshold, and the need for an increased cutoff limit to accomplish clinical usefulness for FIDIS and ELISA, has also been demonstrated by others[35]. At the manufacturer's cutoff levels, FIDIS, EliA, and EUROLINE

all detected higher numbers of anti-dsDNA–positive RA and pSS cases compared to CLIFT, and with regard to this, our results are not entirely in line with a previous study showing a specificity of EliA that was comparable to CLIFT[34].

While in our study the anti-dsDNA levels recorded with CLIFT showed fair correlations and reasonable concordance rates versus FIDIS (rho = 0.62, 84% concordance) and EUROLINE (rho = 0.56, 81%), but somewhat lower ones for EliA (rho = 0.48, 76%), quantification of anti-dsDNA with CLIFT and EUROLINE performed poorly regarding the ability to reflect increases in disease activity among patients with SLE followed over time. We also compared

the 178 cross-sectional SLE sera regarding correlation with disease activity measures, and apart from EUROLINE, significant positive correlations were seen between anti-dsDNA levels and some disease activity variables. However, this was not recorded by the SLEDAI-2K or by the PGA. FIDIS displayed the highest correlation with almost all disease activity variables investigated and was also superior in reflecting disease activity over time as determined by the number of patients with an increase in anti-dsDNA level above 25% between lowest and highest disease activity, and visual interpretation of individual patients over time.

A clear difference between the assays was demonstrated in the negligible overlap of anti-dsDNA–positive disease controls. This could indicate discrepancies in cross-reactivity among the assays, and also suggests that the disease controls were true non-SLE patients. In contrast to EUROLINE and EliA, FIDIS did not identify any of the healthy controls, and the low disease specificity at manufacturer's cutoff was thus a result of a high number of anti-dsDNA–positive patients with RA and pSS. This could speculatively indicate an interference of the antigen with other autoantibodies, because RA and pSS are both autoimmune diseases.

The differences between assays also became apparent in the relation between anti-dsDNA and disease phenotypes defined as ACR-82 criteria. In patients with photosensitivity, there was a decreased risk of anti-dsDNA positivity by CLIFT and FIDIS only, a risk that became even more pronounced for CLIFT when summarizing the skin criteria (ACR criteria 1, 2, or 3). Surprisingly, renal disorder was not significantly associated with CLIFT, but only with FIDIS and EliA. Although the fulfilled ACR criteria only indicate the general disease phenotype, and not necessarily manifestations at blood sampling, these results reflect that anti-dsDNA antibodies are heterogeneous and identified differently depending on assay. The nature of the antigen and its immobilization, as well as other experimental details (e.g., buffers and incubation times), are examples of factors that can affect the detection of autoantibodies. The importance of antigen source and immobilization can be demonstrated by the observation that EliA and FIDIS displayed the highest correlation and concordance (at increased cutoff) of the methods evaluated in our study. Both of these assays use recombinant circular plasmid dsDNA as antigen, in contrast to CLIFT and EUROLINE. The difference seen between EliA and FIDIS (i.e., phenotype specificity) is therefore more likely explained by other factors. One confounding factor could be interfering heterophilic antibodies and autoantibodies, most notably RF, which can disturb an assay by its binding to the Fc parts of other autoantibodies. Such interference could possibly explain the differences between FIDIS and EliA because they differed greatly in their detection of patients with RA, with anti-dsDNA detected by

FIDIS being significantly associated with anti-CCP as well as RF at manufacturer's cutoff, whereas EliA was not.

CLIFT and FIDIS are found to be the best assays for diagnostic purposes given the adjusted cutoff for FIDIS. Based on our findings, FIDIS can also be recommended to monitor disease activity, and could thus potentially replace CLIFT if 1 method for both diagnostics and monitoring of disease activity is preferred. Although the SLICC-12 classification criteria improve the usefulness of ELISA for the determination of anti-dsDNA by their demand for an increased cutoff, an assay-independent recommendation for cutoff limits (i.e., a cutoff based on anti-dsDNA levels in a reference population) would make these criteria even more useful.

REFERENCES
1. Rahman A, Isenberg DA. Systemic lupus erythematosus. N Engl J Med 2008;358:929-39.
2. Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. Arthritis Rheum 1982;25:1271-7.
3. Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. Arthritis Rheum 2012;64:2677-86.
4. Isenberg DA, Manson JJ, Ehrenstein MR, Rahman A. Fifty years of anti-ds DNA antibodies: are we approaching journey's end? Rheumatology 2007;46:1052-6.
5. Tan EM, Schur PH, Carr RI, Kunkel HG. Deoxybonucleic acid (DNA) and antibodies to DNA in the serum of patients with systemic lupus erythematosus. J Clin Invest 1966;45:1732-40.
6. Krishnan C, Kaplan MH. Immunopathologic studies of systemic lupus erythematosus. II. Antinuclear reaction of gamma-globulin eluted from homogenates and isolated glomeruli of kidneys from patients with lupus nephritis. J Clin Invest 1967;46:569-79.
7. Koffler D, Schur PH, Kunkel HG. Immunological studies concerning the nephritis of systemic lupus erythematosus. J Exp Med 1967;126:607-24.
8. Vlahakos DV, Foster MH, Adams S, Katz M, Ucci AA, Barrett KJ, et al. Anti-DNA antibodies form immune deposits at distinct glomerular and vascular sites. Kidney Int 1992;41:1690-700.
9. Aarden LA, de Groot ER, Feltkamp TE. Immunology of DNA. III. Crithidia luciliae, a simple substrate for the determination of anti-dsDNA with the immunofluorescence technique. Ann N Y Acad Sci 1975;254:505-15.
10. Kavanaugh AF, Solomon DH; American College of Rheumatology Ad Hoc Committee on Immunologic Testing Guidelines. Guidelines for immunologic laboratory testing in the rheumatic diseases: anti-DNA antibody tests. Arthritis Rheum 2002;47:546-55.
11. Irving KS, Sen D, Tahir H, Pilkington C, Isenberg DA. A comparison of autoimmune liver disease in juvenile and adult

populations with systemic lupus erythematosus–a retrospective review of cases. Rheumatology 2007;46:1171-3.

12. Czaja AJ, Cassani F, Catateta M, Valentini P, Bianchi FB. Frequency and significance of antibodies to actin in type 1 autoimmune hepatitis. Hepatology 1996;24:1068-73.

13. Eriksson C, Kokkonen H, Johansson M, Hallmans G, Wadell G, Rantapää-Dahlqvist S. Autoantibodies predate the onset of systemic lupus erythematosus in northern Sweden. Arthritis Res Ther 2011;13:R30.

14. Winfield JB, Faiferman I, Koffler D. Avidity of anti-DNA antibodies in serum and IgG glomerular eluates from patients with systemic lupus erythematosus. Association of high avidity antinative DNA antibody with glomerulonephritis. J Clin Invest 1977;59:90-6.

15. Cervera R, Khamashta MA, Font J, Sebastiani GD, Gil A, Lavilla P, et al. Systemic lupus erythematosus: clinical and immunologic patterns of disease expression in a cohort of 1,000 patients. The European Working Party on Systemic Lupus Erythematosus. Medicine 1993;72:113-24.

16. Julkunen H, Ekblom-Kullberg S, Miettinen A. Nonrenal and renal activity of systemic lupus erythematosus: a comparison of two anti-C1q and five anti-dsDNA assays and complement C3 and C4. Rheumatol Int 2012;32:2445-51.

17. Haugbro K, Nossent JC, Winkler T, Figenschau Y, Rekvig OP. Anti-dsDNA antibodies and disease classification in antinuclear antibody positive patients: the role of analytical diversity. Ann Rheum Dis 2004;63:386-94.

18. Launay D, Schmidt J, Lepers S, Mirault T, Lambert M, Kyndt X, et al. Comparison of the Farr radioimmunoassay, 3 commercial enzyme immunoassays and Crithidia luciliae immunofluorescence test for diagnosis and activity assessment of systemic lupus erythematosus. Clin Chim Acta 2010;411:959-64.

19. Eaton RB, Schnneider G, Schur PH. Enzyme immunoassay for antibodies to native DNA. Specificity and quality of antibodies. Arthritis Rheum 1983;26:52-62.

20. Isenberg DA, Dudeney C, Williams W, Addison I, Charles S, Clarke J, et al. Measurement of anti-DNA antibodies: a reappraisal using five different methods. Ann Rheum Dis 1987;46:448-56.

21. Enocsson H, Wetterö J, Skogh T, Sjöwall C. Soluble urokinase plasminogen activator receptor levels reflect organ damage in systemic lupus erythematosus. Transl Res 2013;162:287-96.

22. Frodlund M, Dahlström O, Kastbom A, Skogh T, Sjöwall C. Associations between antinuclear antibody staining patterns and clinical features of systemic lupus erythematosus: analysis of a regional Swedish register. BMJ Open 2013;3:e003608.

23. Gladman DD, Ibañez D, Urowitz MB. Systemic lupus erythematosus disease activity index 2000. J Rheumatol 2002;29:288-91.

24. Scott DL. A simple index to assess disease activity in rheumatoid arthritis. J Rheumatol 1993;20:582-4.

25. Vitali C, Bombardieri S, Jonsson R, Moutsopoulos HM, Alexander EL, Carsons SE, et al. Classification criteria for Sjögren's syndrome: a revised version of the European criteria proposed by the American-European Consensus Group. Ann Rheum Dis 2002;61:554-8.

26. Kastbom A, Strandberg G, Lindroos A, Skogh T. Anti-CCP antibody test predicts the disease course during 3 years in early rheumatoid arthritis (the Swedish TIRA project). Ann Rheum Dis 2004;63:1085-9.

27. Enocsson H, Sjöwall C, Skogh T, Eloranta ML, Rönnblom L, Wetterö J. Interferon-alpha mediates suppression of C-reactive protein: explanation for muted C-reactive protein response in lupus flares? Arthritis Rheum 2009;60:3755-60.

28. Cederblad B, Blomberg S, Vallin H, Perers A, Alm GV, Rönnblom L. Patients with systemic lupus erythematosus have reduced numbers of circulating natural interferon-alpha- producing cells. J Autoimmun 1998;11:465-70.

29. Bootsma H, Spronk PE, Hummel EJ, de Boer G, ter Borg EJ, Limburg PC, et al. Anti-double stranded DNA antibodies in systemic lupus erythematosus: detection and clinical relevance of IgM-class antibodies. Scand J Rheumatol 1996;25:352-9.

30. Förger F, Matthias T, Oppermann M, Becker H, Helmke K. Clinical significance of anti-dsDNA antibody isotypes: IgG/IgM ratio of anti-dsDNA antibodies as a prognostic marker for lupus nephritis. Lupus 2004;13:36-44.

31. Villalta D, Bizzaro N, Bassi N, Zen M, Gatto M, Ghirardello A, et al. Anti-dsDNA antibody isotypes in systemic lupus erythematosus: IgA in addition to IgG anti-dsDNA help to identify glomerulonephritis and active disease. PLoS One 2013;8:e71458.

32. Hopkinson ND, Jenkinson C, Muir KR, Doherty M, Powell RJ. Racial group, socioeconomic status, and the development of persistent proteinuria in systemic lupus erythematosus. Ann Rheum Dis 2000;59:116-9.

33. Hernando M, González C, Sánchez A, Guevara P, Navajo JA, Papisch W, et al. Clinical evaluation of a new automated anti-dsDNA fluorescent immunoassay. Clin Chem Lab Med 2002;40:1056-60.

34. Antico A, Platzgummer S, Bassetti D, Bizzaro N, Tozzoli R, Villalta D, et al. Diagnosing systemic lupus erythematosus: new-generation immunoassays for measurement of anti-dsDNA antibodies are an effective alternative to the Farr technique and the Crithidia luciliae immunofluorescence test. Lupus 2010;19:906-12.

35. Martin J, Durant C, Rimbert M, Hemont C, Hamidou M, Audrain M. Evaluation of two antibodies against double-stranded DNA assays in discriminating between active and non-active systemic lupus erythematosus: correlation between the cut-off and the specificity. Pathol Biol 2012;60:387-91.