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Complete List of Authors:	Frodlund, Martina Dahlström, Örjan Kastbom, Alf Skogh, Thomas Sjöwall, Christopher; IKE, Linköping university, AIR/Rheumatology
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TITLE PAGE

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Authors: Martina Frodlund, MD[1]; Örjan Dahlström, PhD[2]; Alf Kastbom, MD PhD[1]; Thomas Skogh, MD PhD[1]; Christopher Sjöwall, MD PhD[1]*

1. Rheumatology/AIR, Department of Clinical and Experimental Medicine, Linköping University, Linköping
2. Linneaus Centre HEAD, Swedish Institute for Disability Research, Department of Behavioural Sciences and Learning, Linköping University, Linköping, Sweden

*** Corresponding author:**
Rheumatology Unit, University Hospital, SE-581 85 Linköping, Sweden
E-mail address: christopher.sjowall@liu.se
Telephone +46 10 1032416
Fax: +46 10 1031844

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ABSTRACT

Objective. Antinuclear antibody (ANA) analysis by immunofluorescence (IF) microscopy remains a diagnostic hallmark of systemic lupus erythematosus (SLE). The clinical relevance of ANA fine-specificities in SLE has been addressed repeatedly, whereas studies on IF-ANA staining patterns in relation to disease manifestations are very scarce. This study was done to elucidate whether different staining patterns associate with distinct SLE phenotypes.

Design: Observational cohort study.

Setting: One university hospital rheumatology unit in Sweden.

Participants: The study population consisted of 222 cases (89% women; 93% Caucasians), whereof 178 met ≥4/11 of the 1982 American College of Rheumatology criteria (ACR-82). The remaining 20% had an SLE diagnosis based on positive IF-ANA (HEp-2 cells) and ≥2 typical organ manifestations at the time of diagnosis (Fries' criteria).

Outcome measures: The IF-ANA staining patterns homogenous (H-ANA), speckled (S-ANA), combined H+S (HS-ANA), centromeric (C-ANA), nucleolar±other patterns (N-ANA), and other nuclear patterns (oANA) were related to disease manifestations and laboratory measures. Antigen-specificities were also considered regarding double-stranded DNA (*Crithidia luciliae*) and the following extractable nuclear antigens: SS-A/Ro, SS-B/La, Sm, snRNP, Scl-70 and Jo-1 (immunodiffusion and/or line-blot technique).

Results: 54% of the SLE patients displayed H-ANA, 22% S-ANA, 11% HS-ANA, 9% N-ANA, 1% C-ANA, 2% oANA and 1% were never ANA positive. Staining patterns among patients meeting Fries' criteria alone did not differ from those fulfilling ACR-82. H-ANA was significantly associated with ACR-82 criterion 10 ('immunologic disorder'). S-ANA was inversely associated with arthritis, 'immunologic disorder' and signs of organ damage.

Conclusions. H-ANA is the dominant IF-ANA pattern among Swedish SLE patients, and was found to associate with 'immunologic disorder' according to ACR-82. The second most common pattern, S-ANA, associated negatively with arthritis and organ damage.

ABSTRACT SUMMARY

Article focus:

- The use of IF microscopy to identify ANA was introduced in the early 1950's, and this technique still remains the gold standard for ANA diagnostics when screening for autoimmune diseases such as SLE.
- Different IF-ANA staining patterns arise depending on the nuclear antigens targeted and, to some extent, the nuclear staining patterns can have diagnostic implications.
- Herein, we asked if the IF-ANA staining pattern of well-characterized SLE patients included in a regional Swedish register contain any valuable clinical information regarding distinct SLE phenotypes.

Key messages:

- Regardless of the number of fulfilled ACR-82 criteria, H-ANA was the most common IF-ANA pattern among Swedish SLE patients; and this staining was strongly associated with 'immunologic disorder' and anti-dsDNA antibodies.
- S-ANA was the second most common pattern and associated negatively with arthritis and organ damage. These intriguing findings are novel and need to be replicated.
- As previously shown by others, photosensitivity was significantly associated with anti-SSA antibodies. On the contrary, arthritis was less common among patients with anti-SSA antibodies.

Strengths and limitations of this study:

- The large study population with thoroughly organized data and very few internal missing values constitute the strength of this study.

- Although this study confirmed several known associations between serological findings and clinical features, it did not have the power to allow comparisons with specific types of cutaneous lupus, renal disease, central or peripheral nervous system manifestations, as well as with clinical features not included in the ACR criteria.

INTRODUCTION

The clinical spectrum of systemic lupus erythematosus (SLE) is exceedingly variable with an unpredictable disease course characteristically with episodes of flares and remissions. Ongoing disease exacerbations and cumulative damage/dysfunction over time can significantly interfere with quality of life [1]. Organ systems most commonly involved in SLE include joints, skin, mucous membranes, bone marrow, and kidneys. Despite the considerable differences between SLE patients, the occurrence of antinuclear antibodies (ANA) in serum at the time of diagnosis is a common finding with very few exceptions [2].

An "abnormal titer" of ANA assessed by immunofluorescence (IF) microscopy (IF-ANA) is one of the 11 criteria for SLE according to the 1982 American College of Rheumatology (ACR-82) validated classification criteria [3] as well as the 1997 revised criteria [4]. Also the recently proposed Systemic Lupus International Collaborating Clinics (SLICC) criteria state that an ANA test "above the laboratory reference value" remains a criterion for SLE, but without specifying the method for ANA assessment [5]. Unfortunately, none of the classification grounds state how to define the cut-off level for ANA. Similar to the definition of a positive rheumatoid factor test according to the 1987 ACR classification criteria for rheumatoid arthritis (RA) [6], we advocate a cut-off level of >95th percentile among healthy female blood donors to define an abnormal level of ANA analyzed by indirect IF microscopy utilizing fixed HEp-2 cells as source of nuclear antigens and, importantly, gamma-chain specific secondary antibodies to pinpoint IgG-class IF-ANA [7]. At this cut-off level, ANA has very high diagnostic sensitivity for SLE, but low diagnostic specificity, with close to 5% prevalence among healthy female blood

donors [8]. Accordingly, ANA testing should only be done upon fair clinical indications of ANA-related disease [9].

Nuclear constituents such as histone proteins, double-stranded (ds) DNA, DNA/histone complexes (nucleosomes), various nuclear enzymes and other proteins/ribonucleoproteins are common target antigens for ANA. On the basis of their different intra-nuclear distributions, IF-ANA staining patterns can be subdivided into homogenous /chromosomal (H-ANA), centromeric (C-ANA), speckled/extrachromosomal (S-ANA), nucleolar (N-ANA), nuclear membrane, nuclear dot and other defined patterns [10]. We previously reported that the H-ANA is the most common among SLE patients from southern Sweden, but also the most common amongst ANA positive healthy persons as well as in RA [8]. Antibodies against dsDNA, histones and DNA/histone complex all yield an H-ANA pattern [11]. The presence of anti-dsDNA, which is included in ACR-82 criterion number 10 designated 'immunologic disorder', has been regarded as a fairly specific diagnostic marker of SLE and is very common in lupus nephritis [2, 10–13]. S-ANA is generated by antibodies targeting 'extractable nuclear antigens' (ENA), *i.e.* a group of extra-chromosomal antigens which are readily extracted with 0.15M sodium chloride, for instance 'small nuclear ribonucleoprotein' (snRNP) and the 'Smith antigen' (Sm), which are both located on U1-RNP particles [2, 10, 11]. Anti-Sm antibody detected by double radial immunodiffusion (DRID) in gel is highly specific for SLE and practically always occurs together with anti-snRNP. Anti-Sm has been reported to associate with constitutional symptoms (pyrexia, weight-loss and fatigue), nephritis and central nervous system disease, but the sensitivity in cohorts worldwide varies dramatically due to ethnicity [11, 14–16]. Anti-Sm has also been reported to associate with serositis and Raynaud's phenomenon [17–

19]. N-ANA patterns are not typical of SLE, but rather of systemic sclerosis of the diffuse type [20]. N-ANA may be directed against *e.g.* fibrillarin, RNA-polymerase 1–3, 'PM-Scl', and Scl-70 (topoisomerase-1) [10, 11, 21]. Scl-70, which belongs to the 'ENA family', is also found both extra-chromosomally in the nucleoplasm and bound to DNA, thus giving rise to a mixed IF staining pattern. Like Scl-70, the SS-B/La antigen may partly localize in nucleoli. Most experience regarding clinical associations to anti-ENA refers to DRID analyses. As regards anti-SSB as well as anti-SSA, a positive DRID test is clinically linked to Sjögren's syndrome and to some extent SLE [17, 22–26]. A positive anti-SSB/DRID test generally occurs together with anti-SSA, whereas anti-SSA is frequently demonstrated in the absence of anti-SSB. Since the concentration of SS-A is low in HEp-2 cells, anti-SSA escapes detection when non-transfected HEp-2 cells are used as ANA substrate for IF microscopy [27]. In a small proportion of pregnant women with circulating anti-SSA/Ro52, transplacental antibody passage to the fetus can result in neonatal lupus, *i.e.* typical congenital skin rash (which vanishes in parallel with elimination of the maternal antibodies) and sometimes also in congenital lifelong complete atrioventricular heart block [28, 29].

Although several studies have dealt with the clinical significance of ANA fine-specificities in SLE, very few have evaluated if/how different IF-ANA staining patterns may relate to distinct clinical lupus features. In the present study we aimed at comparing IF-ANA staining patterns with defined clinical and laboratory disease manifestations among well-characterized cases of SLE.

PATIENTS AND METHODS

Subjects

222 SLE patients (198 women and 24 men; mean age 51 years; range 18–88) taking part in the prospective follow-up programme KLURING (a Swedish acronym for 'Clinical LUpus Register in Northeastern Gothia') at the Rheumatology clinic, Linköping university hospital, Sweden were included between September 2008 and November 2012. This corresponds to about 95% of the expected SLE cases in the catchment area of Linköping and ≥98% of all known SLE cases. The patient material was recently described in detail [30]. 178 patients (80%) met the ACR-82 criteria [3], and 44 (20%) had a clinical diagnosis of SLE based on a history of abnormal ANA titer (specified below), and at least 2 typical organ manifestations at the time of diagnosis (referred to as the Fries' criteria) [31, 32]. Patients were consecutively recruited; most were prevalent cases (85%), but some (15%) had newly diagnosed SLE at the time of enrollment. Distribution of age at disease onset is demonstrated in Figure 1. The median disease duration by year 2012 was 12 years (mean 13.4; range 0–49). Disease severity/organ damage was estimated using the SLICC/ACR damage index (SDI) at the end of year 2011 or from the last observation made [33]. 206 (93%) of the patients were Caucasians. 92 (41%) of the patients were prescribed antimalarials (AM) alone, 68 (31%) other disease-modifying anti-rheumatic drugs ± AM and 128 (58%) oral glucocorticoids. IF-ANA staining patterns, anti-ENA reactivity and dsDNA antibodies were analyzed on a routine basis at the Clinical immunology laboratory, Linköping university hospital and were extracted from medical records. In many patients IF-ANA analysis was performed at several occasions over time, but discrepant staining patterns

were achieved in less than 5% of these cases. Herein, IF-ANA staining pattern from the time-point most adjacent to SLE diagnosis was used for comparisons with clinical and laboratory features.

Indirect IF microscopy

ANA was analyzed by indirect IF microscopy using multispot slides with fixed HEp-2 cells (ImmunoConcepts, Sacramento, CA, USA) as antigen substrate and fluorescein-isothiocyanate (FITC) conjugated gamma-chain specific anti-human IgG as detection antibody (DAKO, Glostrup, Denmark). The cut-off level for a positive ANA test was set at a titer of 1:200, corresponding to >95th percentile among 150 healthy female blood donors. Positive ANA tests were categorized regarding IF staining patterns (H-ANA, S-ANA, HS-ANA, N-ANA ± other pattern, or other staining patterns [here designated oANA]). To qualify as an H-ANA pattern, chromatin staining was required in metaphase/anaphase cells and, likewise, absence of chromatin staining was required to qualify as a pure S-ANA pattern. Microscope slides with fixed *Crithidia luciliae* (ImmunoConcepts) were used to analyze IgG-class anti-dsDNA antibodies by IF with a cut-off titer at 1:10, corresponding to >99th percentile among 100 healthy blood donors.

Anti-ENA antibodies

Autoantibodies to ENA included the following specificities: SSA, SSB, Sm, snRNP, Scl-70 and Jo-1, and were analyzed by DRID (ImmunoConcepts) and/or line-blot technique (ProfilePlus, R052 Euroassay, Euroimmun, Lübeck, Germany). In the case line-blot screening resulted in positive reactions regarding antibodies against Sm, Jo-1 or Scl-70, these specificities were confirmed by DRID in order to qualify as positive. For the other

anti-ENA specificities, good reproducibility has been reassured at the performing laboratory.

Routine laboratory analyses

To assess hematologic and renal disorders, laboratory tests at selected visits included hemoglobin and blood cell counts (erythrocytes, total leukocyte count, lymphocytes, neutrophils and platelets) as well as urinalysis (dip-slide procedure for erythrocytes, protein and glucose), urinary sediment assessment and serum creatinine. Lupus anticoagulant was performed by the dilute Russell's viper venom test (DRVVT).

Renal histopathology

38 of the included patients (*i.e.* 79% of those who fulfilled ACR-82 criterion number 7 'renal disorder') had undergone renal biopsy performed by percutaneous ultrasonography-guided puncture in accordance with a standard protocol. The renal tissue obtained was classified according to the WHO classification for lupus nephritis [34]. All biopsies were evaluated by conventional light microscopy, direct IF and electron microscopy.

Statistics

Frequencies of the different IF-ANA staining patterns in the study group were analyzed to identify subgroups for further analyses. Clinical and laboratory features were described by their frequencies, for each of most common pattern subgroups separately.

Differences in distributions of different staining patterns regarding clinical and laboratory features were analyzed using Chi-square tests of independence (alternatively Fisher's exact test in case of small expected frequencies) with Cramer's V as measure of effect size. All statistics were performed using IBM SPSS 20.0. *P*-values <0.05 were considered significant.

Ethical considerations

Oral and written informed consent was obtained from all subjects. The study protocol was approved by the regional ethics committee in Linköping, Sweden (M75-08/2008).

RESULTS

Frequencies of clinical and laboratory features are displayed in Table 1ab. 219 of 222 (99%) were found to be ever ANA positive. Skin disease and arthritis were the most commonly fulfilled ACR-82 criteria followed by 'hematologic disorder'. 22% of the patients had renal disease and 44% showed positive anti-dsDNA antibody test at least once during their disease course. However, 5 individuals were classified with unknown or other pattern (oANA) since the Clinical immunology laboratory was unable to recover documentation of IF-ANA patterns or classified the positive nuclear staining pattern as very rare (nuclear dots). H-ANA staining was by far the most frequent pattern (54%) followed by S-ANA (22%), HS-ANA (11%), N-ANA ± other pattern (9%) and C-ANA (1%). The first 4 pattern groups were considered large enough for statistical comparisons.

Some clinical and laboratory features showed differences in proportions over different staining patterns (Table 1). 'Immunologic disorder' and anti-dsDNA antibodies were more often associated with H-ANA, and less often associated with S-ANA; whereas anti-snRNP showed the opposite direction (moderate to strong effects). Central nervous system disease was less often associated with H-ANA compared to other staining patterns, but the number of affected individuals was very low. Anti-Sm was more often, whereas arthritis and organ damage (SDI ≥1) respectively were less often, associated with S-ANA. Anti-SSA and anti-SSB antibodies were more often associated with HS-ANA.

Table 1a

Clinical feature (ACR–82)	H-ANA (%) (*n* = 119)	S-ANA (%) (*n* = 49)	HS-ANA (%) (*n* = 24)	N-ANA* (%) (*n* = 19)	*P*-value	Cramer's V	C-ANA (%) (*n* = 3)	oANA (%) (*n* = 5)	Total (%)
Malar rash	42.0	53.1	41.7	31.6	0.38		0	80	43.8
Discoid lupus	12.6	18.4	20.8	10.5	0.57†		33	20	15.1
Photosensitivity	47.9	65.3	58.3	36.8	0.09		33	80	52.5
Oral ulcers	10.1	16.3	12.5	10.5	0.68†		0	0	11.4
Arthritis	76.5	63.3 –	91.7	89.5	0.02	0.23	100	100	77.2
Serositis	42.9	38.8	25.0	47.4	0.38		100	20	40.6
Pleuritis	38.7	34.7	25.0	36.8	0.64		100	20	36.5
Pericarditis	16.0	14.3	0.0	15.8	0.15†		33	0	13.7
Renal disorder	24.4	16.3	29.2	15.8	0.49		33	0	21.9
Neurologic disorder	1.7 –	8.2	8.3	10.5	0.04†	0.17	33	0	5.0
Seizures	0.8 –	6.1	8.3	10.5	0.02†	0.19	33	0	4.1
Psychosis	0.8	2.0	0.0	5.3	0.22†		0	0	1.4
Hematologic disorder	48.7	59.2	58.3	42.1	0.45		33	0	50.2
Immunologic disorder	64.7 +	24.5 –	33.3	31.6	<0.001	0.37	33	0	47.5
Antinuclear antibody	100	100	100	100			100	100	100
≥ 6 fulfilled ACR criteria	26.9	24.5	20.8	15.8	0.73		33	0	24.2
SDI score ≥ 1	59.7	30.6 –	54.2	57.9	0.007	0.24	67	60	52.5

H = Homogenous. S = Speckled, HS = Homogenous/Speckled, N = Nucleolar, C = Centromeric, oANA = other pattern, + = positive association, – = negative association.

* Staining pattern ± combination with other pattern(s).

† Fisher's exact test.

Table 1b

Laboratory feature	H-ANA (%) (*n* = 119)	S-ANA (%) (*n* = 49)	HS-ANA (%) (*n* = 24)	N-ANA* (%) (*n* = 19)	*P*-value	Cramer's V	C-ANA (%) (*n* = 3)	oANA (%) (*n* = 5)	Total (%)
Hemolytic anemia	2.5	8.2	4.2	5.3	0.30†		0	0	4.1
Leukocytopenia	29.4	30.6	33.3	21.1	0.84		33	0	28.8
Lymphocytopenia	27.7	32.7	33.3	31.6	0.90		0	0	28.8
Thrombocytopenia	10.1	16.3	12.5	5.3	0.59†		0	0	11.0
Lupus anticoagulant#	34.6	24.3	33.3	38.5	0.68		33	50	32.5
Anti-dsDNA	63.9 +	12.2 –	33.3	26.3	<0.001	0.45	33	0	43.8
Anti-Sm	3.4	16.7 +	4.2	10.5	0.022†	0.21	0	0	7.0
Anti-SSA/Ro	32.8	43.8	62.5 +	36.8	0.047	0.20	33	0	38.5
Anti-SSB/La	7.0	12.8	33.3 +	0	0.002†	0.29	0	0	11.8
Anti-snRNP	6.9 –	47.8 +	13.6	22.2	<0.001†	0.43	0	0	20.2

H = Homogenous. S = Speckled, HS = Homogenous/Speckled, N = Nucleolar, C = Centromeric, oANA = other pattern, + = positive association, – = negative association.

* Staining pattern ± combination with other pattern(s).

† Fisher's exact test.

Not analyzed in all patients; H-ANA: *n* = 81, S-ANA: *n* = 37, HS-ANA: *n* = 18, N-ANA: *n* = 13, C-ANA: *n* = 3, oANA: *n* = 2.

Photosensitivity was significantly associated with anti-SSA antibodies (Figure 2). On the contrary, arthritis was less common among patients with anti-SSA antibodies. A positive anti-Sm antibody test was significantly associated with lymphocytopenia (Fisher's exact test, p =0.014, Cramer's V =0.19); and as expected, a positive anti-dsDNA antibody test was significantly associated with renal disorder (Chi-square test, p <0.001, Cramer's V =0.34).

The proportions of different staining patterns in the group of patients fulfilling only the Fries' criteria and those meeting the ACR-82 criteria are demonstrated in Figure 3. The higher proportion of patients with nucleolar staining in the Fries' group as compared to the ACR-82 group did not meet statistical significance (Fisher's exact test, p =0.064). Figure 4 demonstrates the number of fulfilled ACR criteria in relation to nuclear staining patterns. H-ANA was found to dominate regardless of the number of fulfilled ACR criteria. As indicated in Figure 5, H-ANA was significantly more common in patients that had been classified with proliferative lupus nephritis (WHO class 3 or 4) on renal biopsy (Chi-square test, p <0.001) compared to other staining patterns.

DISCUSSION

The use of IF microscopy to identify antinuclear antibodies was introduced by Holman, Kunkel and Friou already in the early 1950's [35, 36], and still remains the gold standard for ANA diagnostics [9, 37]. Different IF-ANA staining patterns arise depending on the nuclear antigens targeted and, to some extent, the nuclear staining patterns can have diagnostic implications [10, 11]. Furthermore, being an exceptionally heterogeneous disease entity, different SLE phenotypes may associate with different ANA subspecificities. Nevertheless, studies on IF-ANA staining patterns in relation to SLE subtypes are very scarce. Thus, herein we asked if the IF-ANA staining pattern of well-characterized SLE patients in a regional Swedish register *per se* contain any valuable clinical information.

In a previous investigation based on South Swedish SLE patients who had all been judged IF-ANA positive at the time-point of diagnosis, a considerable proportion (24%) lost their ANA positivity over time [8]. This may appear surprising, but our findings are very consistent with the results from a recent clinical trial for belimumab in SLE [38]. Our study demonstrated that, among those remaining IF-ANA positive over time, the vast majority (62%) displayed H-ANA ± other pattern, whereas fewer had a pure S-ANA pattern (10%). In the present study, we confirmed that H-ANA is the most common IF-ANA pattern among Swedish SLE patients regardless of the number of fulfilled ACR criteria. The fact that we did not find any significant difference between ANA staining patterns in patients fulfilling the ACR-82 classification criteria and those that only met

Fries' criteria probably reflects that the ACR-82 criteria have a lower sensitivity and fail to identify all patients with 'clinical SLE' and at least 2 typical organ manifestations.

Many have dealt with differences in ANA fine-specificity and grouped patients according to ANA seroprofiles in order to reveal potential associations with defined clinical lupus manifestations [14–19, 22–26, 39, 40]. Using the luciferase immunoprecipitation system, Ching *et al.* recently reported that the anti-Sm/snRNP-cluster was more associated with serositis than with the anti-SSA/SSB cluster [19]. Thompson and colleagues observed that SLE cases with anti-dsDNA and/or anti-Sm were more likely to have malar rash, hypocomplementemia, renal and hematologic involvement than patients without these autoantibodies [17]. Several studies have also concluded that anti-SSA positive patients have an increased rate of lupus-related rash and photosensitivity [17, 22, 24]. Thus, our finding of a significant association between anti-SSA and photosensitivity was expected.

In a recent and very large study from China, 1928 SLE patients from 5 different centers were studied according to serological profiles [41]. The presence of anti-dsDNA was found to be associated with renal disorder, serositis and hematological involvement. In our study, anti-dsDNA was exclusively associated with renal disorder. Only 15% of the Chinese lupus cohort exhibited the anti-Sm/snRNP/phospholipid-cluster, but these patients had the highest frequency of malar rash, oral ulcers, arthritis and serositis [41]. As expected, skin disease/photosensitivity was associated with the anti-SSA/SSB cluster, but contrasting to our findings Li *et al.* reported a positive association between anti-SSA and arthritis. The reason for the contradictory findings may be sought in differences in methodology as well as in genetic factors.

Organ damage is strongly connected to SLE prognosis [42, 43], but only one biomarker (osteopontin) has so far been shown to predict organ damage [44]. In the present study, organ damage (SDI ≥1) was significantly less common among patients with S-ANA. This is a novel finding which calls for confirmation by others. A plausible explanation is that anti-dsDNA antibodies were also less common among cases with S-ANA and, given the strong association between anti-dsDNA and lupus nephritis [13, 16], patients with S-ANA may have less (or at least milder) renal disease with a subsequent risk of developing organ damage. Another possible explanation is the well-documented association between anti-SSA/SSB and milder disease manifestations, *e.g.* lupus-related rash and photosensitivity [17, 22, 24]. Importantly, however, anti-SSA antibodies are not visualized on standard HEp-2 cells (used in this study) since the antigen levels are low.

To conclude, the results of this study demonstrate that IF-ANA staining patterns have some clinical correlates of potential diagnostic and prognostic interest in addition to traditional antigen-specific immunoassays. The findings that arthritis and signs of organ damage were less often associated with S-ANA compared to other staining patterns call for confirmatory studies and further elaborations, including identification of ANA fine-specificities.

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Contributors. MF was involved in conception and design of the study, data collection and manuscript writing. ÖD contributed with statistical advice, interpretation of data and drafted the paper. AK was involved in acquisition of patient data, interpretation of data, intellectual discussion and manuscript writing. TS was involved in the laboratory work, interpretation of data, intellectual discussion and drafted the paper. CS contributed to the original idea, patient characterization, interpretation of data, intellectual discussion and manuscript writing.

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Competing interests. None.

Ethics approval. The regional ethics committee in Linköping, Sweden.

Data sharing. No additional data are available.

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FIGURE LEGENDS

Figure 1: Percent of SLE patients by sex and decade of age at disease onset.

Figure 2: Percentage of patients fulfilling ACR-82 criterion 3 (photosensitivity) and 5 (arthritis) in relation to anti-SSA antibody status. Photosensitivity was significantly more common, and arthritis less common, in anti-SSA antibody positive SLE patients. Data on anti-SSA antibody status was available in 216 of 222 (97.3%) cases.

Figure 3: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on those who only met the Fries' criteria and those who fulfilled at least 4 out of the 11 ACR-82 criteria.

Figure 4: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on the number of fulfilled ACR-82 criteria.

Figure 5: IF-ANA staining patterns demonstrated for the 38 patients that had undergone renal biopsy divided according to the WHO classification for lupus nephritis.

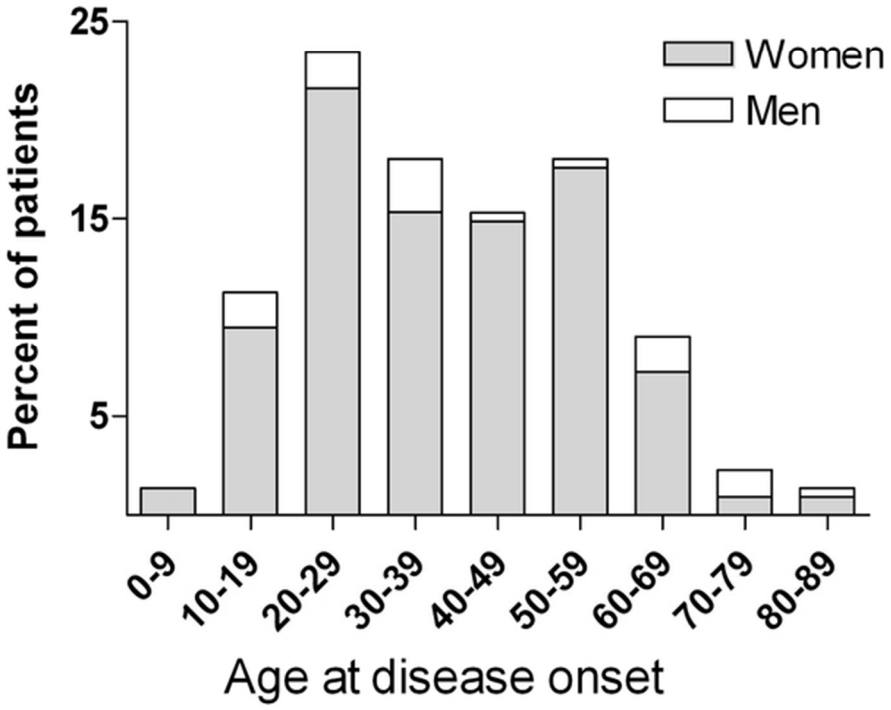

Figure 1: Percent of SLE patients by sex and decade of age at disease onset.
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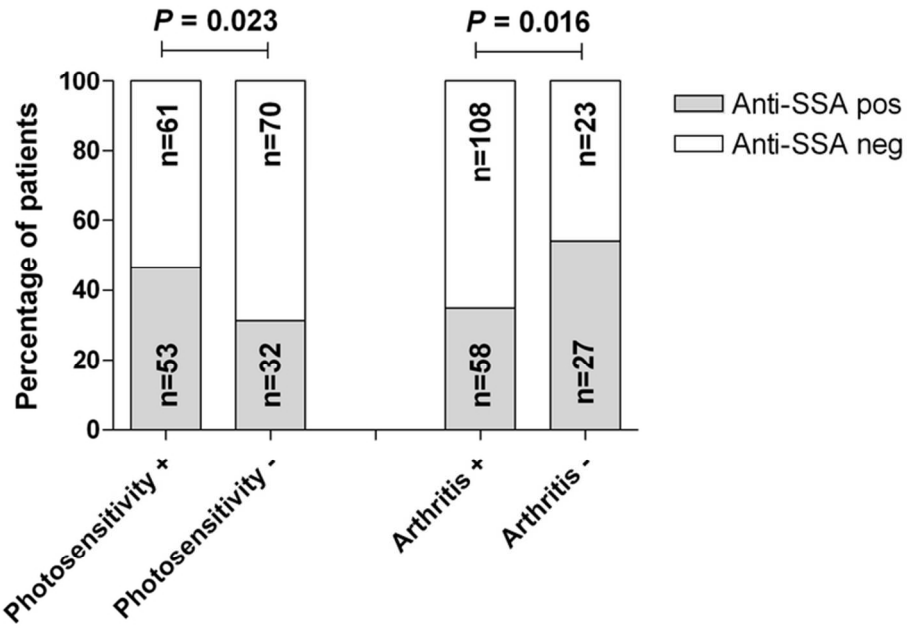

Figure 2: Percentage of patients fulfilling ACR-82 criterion 3 (photosensitivity) and 5 (arthritis) in relation to anti-SSA antibody status. Photosensitivity was significantly more common, and arthritis less common, in anti-SSA antibody positive SLE patients. Data on anti-SSA antibody status was available in 216 of 222 (97.3%) cases.
71x48mm (300 x 300 DPI)

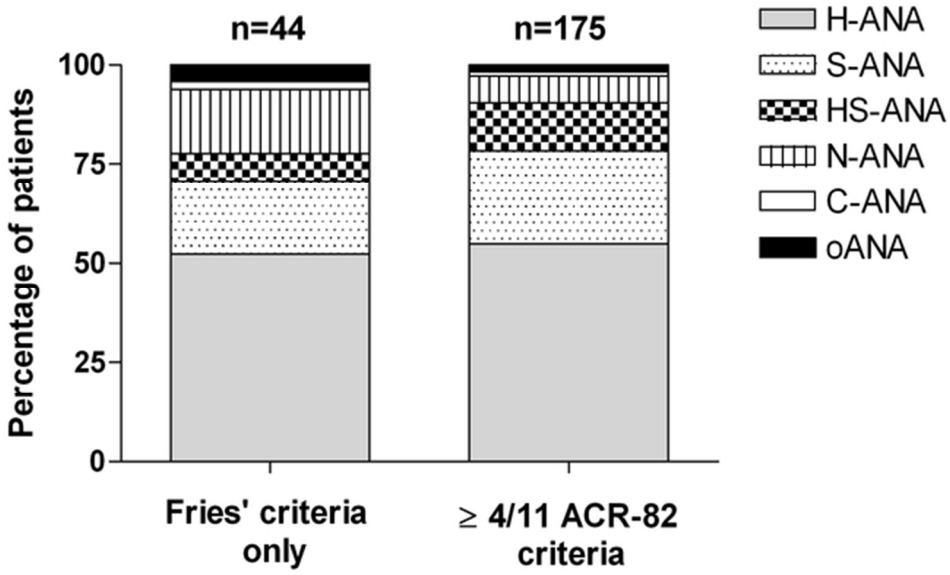

Figure 3: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on those who only met the Fries' criteria and those who fulfilled at least 4 out of the 11 ACR-82 criteria.
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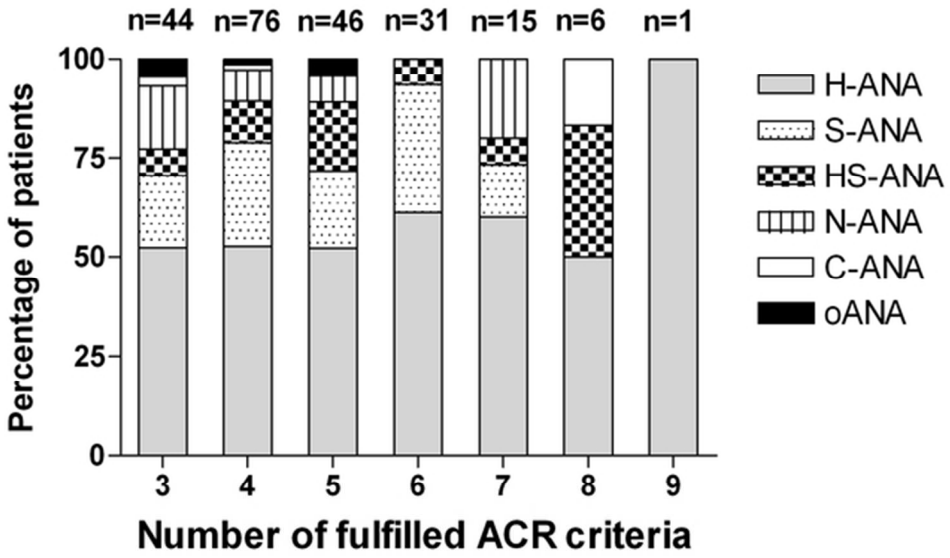

Figure 4: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on the number of fulfilled ACR-82 criteria.
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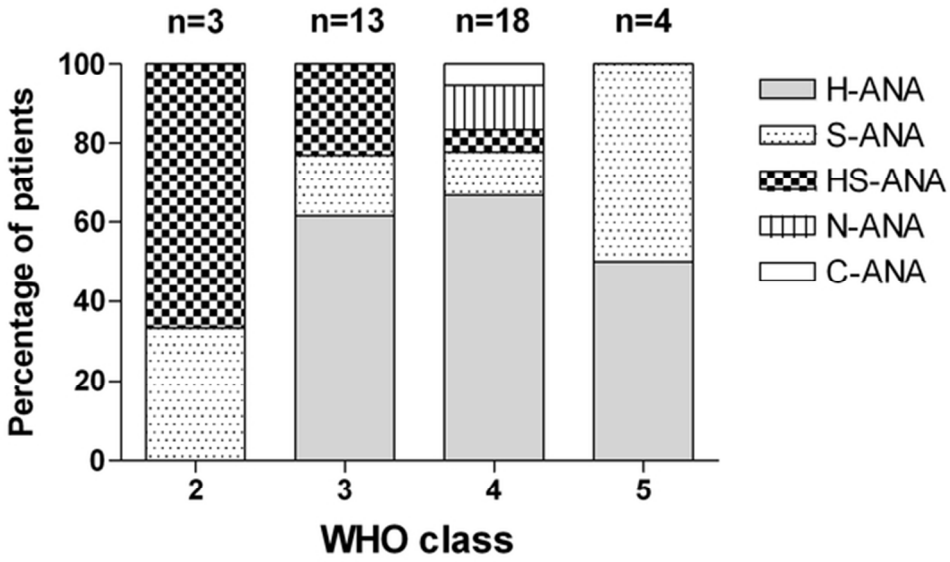

Figure 5: IF-ANA staining patterns demonstrated for the 38 patients that had undergone renal biopsy divided according to the WHO classification for lupus nephritis.
57x35mm (300 x 300 DPI)

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Associations between antinuclear antibody staining patterns and clinical features of systemic lupus erythematosus: analysis of a regional Swedish register

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TITLE PAGE

Manuscript title: Associations between antinuclear antibody staining patterns and clinical features of systemic lupus erythematosus: analysis of a regional Swedish register

Authors: Martina Frodlund, MD[1]; Örjan Dahlström, PhD[2]; Alf Kastbom, MD PhD[1]; Thomas Skogh, MD PhD[1]; Christopher Sjöwall, MD PhD[1]*

1. Rheumatology/AIR, Department of Clinical and Experimental Medicine, Linköping University, Linköping
2. Linneaus Centre HEAD, Swedish Institute for Disability Research, Department of Behavioural Sciences and Learning, Linköping University, Linköping, Sweden

*** Corresponding author:**
Rheumatology Unit, University Hospital, SE-581 85 Linköping, Sweden
E-mail address: christopher.sjowall@liu.se
Telephone +46 10 1032416
Fax: +46 10 1031844

Running head: ANA staining patterns *versus* SLE phenotypes

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ABSTRACT

Objective. Antinuclear antibody (ANA) analysis by immunofluorescence (IF) microscopy remains a diagnostic hallmark of systemic lupus erythematosus (SLE). The clinical relevance of ANA fine-specificities in SLE has been addressed repeatedly, whereas studies on IF-ANA staining patterns in relation to disease manifestations are very scarce. This study was done to elucidate whether different staining patterns associate with distinct SLE phenotypes.

Design: Observational cohort study.

Setting: One university hospital rheumatology unit in Sweden.

Participants: The study population consisted of 222 cases (89% women; 93% Caucasians), whereof 178 met ≥4/11 of the 1982 American College of Rheumatology criteria (ACR-82). The remaining 20% had an SLE diagnosis based on positive IF-ANA (HEp-2 cells) and ≥2 typical organ manifestations at the time of diagnosis (Fries' criteria).

Outcome measures: The IF-ANA staining patterns homogenous (H-ANA), speckled (S-ANA), combined H+S (HS-ANA), centromeric (C-ANA), nucleolar±other patterns (N-ANA), and other nuclear patterns (oANA) were related to disease manifestations and laboratory measures. Antigen-specificities were also considered regarding double-stranded DNA (*Crithidia luciliae*) and the following extractable nuclear antigens: Ro/SSA, La/SSB, Sm, snRNP, Scl-70 and Jo-1 (immunodiffusion and/or line-blot technique).

Results: 54% of the SLE patients displayed H-ANA, 22% S-ANA, 11% HS-ANA, 9% N-ANA, 1% C-ANA, 2% oANA and 1% were never ANA positive. Staining patterns among patients meeting Fries' criteria alone did not differ from those fulfilling ACR-82. H-ANA was significantly associated with the 10th criterion according to ACR-82 ('immunologic disorder'). S-ANA was inversely associated with arthritis, 'immunologic disorder' and signs of organ damage.

Conclusions. H-ANA is the dominant IF-ANA pattern among Swedish SLE patients, and was found to associate with 'immunologic disorder' according to ACR-82. The second most common pattern, S-ANA, associated negatively with arthritis and organ damage.

ABSTRACT SUMMARY

Article focus:

- The use of IF microscopy to identify ANA was introduced in the early 1950's, and this technique still remains the gold standard for ANA diagnostics when screening for autoimmune diseases such as SLE.
- Different IF-ANA staining patterns arise depending on the nuclear antigens targeted and, to some extent, the nuclear staining patterns can have diagnostic implications.
- Herein, we asked if the IF-ANA staining pattern of well-characterized SLE patients included in a regional Swedish register contain any valuable clinical information regarding distinct SLE phenotypes.

Key messages:

- Regardless of the number of fulfilled ACR-82 criteria, H-ANA was the most common IF-ANA pattern among Swedish SLE patients; and this staining was strongly associated with 'immunologic disorder' and anti-dsDNA antibodies.
- S-ANA was the second most common pattern and associated negatively with arthritis and organ damage. These intriguing findings are novel and need to be replicated.
- As previously shown by others, photosensitivity was significantly associated with anti-Ro/SSA antibodies. On the contrary, arthritis was less common among patients with anti-Ro/SSA antibodies.

Strengths and limitations of this study:

- The large study population with thoroughly organized data and very few internal missing values constitute the strength of this study.

- Although this study confirmed several known associations between serological findings and clinical features, it did not have the power to allow comparisons with specific types of cutaneous lupus, renal disease, central or peripheral nervous system manifestations, as well as with clinical features not included in the ACR criteria.

INTRODUCTION

The clinical spectrum of systemic lupus erythematosus (SLE) is exceedingly variable with an unpredictable disease course characteristically with episodes of flares and remissions. Ongoing disease exacerbations and cumulative damage/dysfunction over time can significantly interfere with quality of life [1]. Organ systems most commonly involved in SLE include joints, skin, mucous membranes, bone marrow, and kidneys. Despite the considerable differences between SLE patients, the occurrence of antinuclear antibodies (ANA) in serum at the time of diagnosis is a common finding with very few exceptions [2].

An "abnormal titer" of ANA assessed by immunofluorescence (IF) microscopy (IF-ANA) is one of the 11 criteria for SLE according to the 1982 American College of Rheumatology (ACR-82) validated classification criteria [3] as well as the 1997 revised criteria [4]. Also the recently proposed Systemic Lupus International Collaborating Clinics (SLICC) criteria state that an ANA test "above the laboratory reference value" remains a criterion for SLE, but without specifying the method for ANA assessment [5]. Unfortunately, none of the classification grounds state how to define the cut-off level for ANA. Similar to the definition of a positive rheumatoid factor test according to the 1987 ACR classification criteria for rheumatoid arthritis (RA) [6], we advocate a cut-off level of >95th percentile among healthy female blood donors to define an abnormal level of ANA analyzed by indirect IF microscopy utilizing fixed HEp-2 cells as source of nuclear antigens and, importantly, gamma-chain specific secondary antibodies to pinpoint IgG-class IF-ANA [7]. At this cut-off level, ANA has very high diagnostic sensitivity for SLE, but low diagnostic specificity, with close to 5% prevalence among healthy female blood

donors [8]. Accordingly, ANA testing should only be done upon fair clinical indications of ANA-related disease. Although circulating levels of ANA may vary over time in SLE patients, the correlation between IF-ANA titer and clinical activity is poor [9].

Nuclear constituents such as histone proteins, double-stranded (ds) DNA, DNA/histone complexes (nucleosomes), various nuclear enzymes and other proteins/ribonucleoproteins are common target antigens for ANA. On the basis of their different intra-nuclear distributions, IF-ANA staining patterns can be subdivided into homogenous /chromosomal (H-ANA), centromeric (C-ANA), speckled/extrachromosomal (S-ANA), nucleolar (N-ANA), nuclear membrane, nuclear dot and other defined patterns [10]. The most common ANA pattern detected among healthy individuals has been reported as a uniformly distributed staining of HEp-2 cells in the interphase and a chromosomal staining in dividing cells, designated 'dense fine speckled pattern', whereas we have actually referred to this staining as a homogenous/chromosomal pattern, *i.e.* H-ANA [8, 11, 12]. A 'classical' homogenous/chromosomal pattern is the most common among SLE patients from southern Sweden as well as in RA [8]. Antibodies against dsDNA, histones and DNA/histone complex all yield a 'classical' H-ANA pattern on HEp-2 cells [13]. Antibodies against dsDNA, histones and DNA/histone complex all yield an H-ANA pattern [13]. The presence of anti-dsDNA, which is included in ACR-82 criterion number 10 designated 'immunologic disorder', has been regarded as a fairly specific diagnostic marker of SLE and is very common in lupus nephritis [2, 10, 13–15]. S-ANA is generated by antibodies targeting 'extractable nuclear antigens' (ENA), *i.e.* a group of extra-chromosomal antigens which are readily extracted with 0.15M sodium chloride, for instance 'small nuclear ribonucleoprotein' (snRNP) and the 'Smith antigen' (Sm), which

are both located on U1-RNP particles [2, 10, 13]. Anti-Sm antibody detected by double radial immunodiffusion (DRID) in gel is highly specific for SLE and practically always occurs together with anti-snRNP. Anti-Sm has been reported to associate with constitutional symptoms (pyrexia, weight-loss and fatigue), nephritis and central nervous system disease, but the sensitivity in cohorts worldwide varies dramatically due to ethnicity [13, 17–21]. Anti-Sm has also been reported to associate with serositis and Raynaud's phenomenon [19, 22–24]. N-ANA patterns are not typical of SLE, but rather of systemic sclerosis of the diffuse type [25]. N-ANA may be directed against *e.g.* fibrillarin, RNA-polymerase 1–3, 'PM-Scl', and Scl-70 (topoisomerase-1) [10, 13, 26]. Scl-70, which belongs to the 'ENA family', is also found both extra-chromosomally in the nucleoplasm and bound to DNA, thus giving rise to a mixed IF staining pattern. Like Scl-70, the La/SSB antigen may partly localize in nucleoli. Most experience regarding clinical associations to anti-ENA refers to DRID analyses. As regards anti-La/SSB as well as anti-Ro/SSA, a positive DRID test is clinically linked to Sjögren's syndrome and to some extent SLE [22, 27–31]. A positive anti-La/SSB DRID test generally occurs together with anti-Ro/SSA, whereas anti-Ro/SSA is frequently demonstrated in the absence of anti-La/SSB. Since the concentration of Ro/SSA is low in HEp-2 cells, anti-Ro/SSA escapes detection when non-transfected HEp-2 cells are used as ANA substrate for IF microscopy [32]. In a small proportion of pregnant women with circulating anti-Ro52/SSA, transplacental antibody passage to the fetus can result in neonatal lupus, *i.e.* typical congenital skin rash (which vanishes in parallel with elimination of the maternal antibodies) and sometimes also in congenital lifelong complete atrioventricular heart block [33, 34].

Although several studies have dealt with the clinical significance of ANA fine-specificities in SLE, very few have evaluated if/how different IF-ANA staining patterns may relate to distinct clinical lupus features. In the present study we aimed at comparing IF-ANA staining patterns with defined clinical and laboratory disease manifestations among well-characterized cases of SLE.

PATIENTS AND METHODS

Subjects

222 SLE patients (198 women and 24 men; mean age 51 years; range 18–88) taking part in the prospective follow-up programme KLURING (a Swedish acronym for 'Clinical LUpus Register in Northeastern Gothia') at the Rheumatology clinic, Linköping university hospital, Sweden were included between September 2008 and November 2012. This corresponds to about 95% of the expected SLE cases in the catchment area of Linköping and ≥98% of all known SLE cases. The patient material was recently described in detail [35]. 178 patients (80%) met the ACR-82 criteria [3], and 44 (20%) had a clinical diagnosis of SLE based on a history of abnormal ANA titer (specified below), and at least 2 typical organ manifestations at the time of diagnosis (referred to as the Fries' criteria) [36, 37]. The presence of anti-cardiolipin antibodies of IgG and/or IgM class detected by ELISA and/or positive lupus anticoagulant test (not classified as an immunologic criterion according to ACR-82) was found in 31 of the 44 individuals (70%) in the Fries group.

Patients were consecutively recruited; most were prevalent cases (85%), but some (15%) had newly diagnosed SLE at the time of enrollment. Distribution of age at disease onset is demonstrated in Figure 1. The median disease duration by year 2012 was 12 years (mean 13.4; range 0–49). Disease severity/organ damage was estimated using the SLICC/ACR damage index (SDI) at the end of year 2011 or from the last observation made [38]. 206 (93%) of the patients were Caucasians. 92 (41%) of the patients were prescribed antimalarials (AM) alone, 68 (31%) other disease-modifying anti-rheumatic

drugs ± AM and 128 (58%) oral glucocorticoids. IF-ANA staining patterns, anti-ENA reactivity and dsDNA antibodies were analyzed on a routine basis at the Clinical immunology laboratory, Linköping university hospital and were extracted from medical records. In many patients, IF-ANA analysis was performed at several occasions over time but discrepant staining patterns were achieved in less than 5% of these cases. Herein, IF-ANA staining pattern from the time-point most adjacent to SLE onset was used for comparisons with clinical and laboratory features.

Indirect IF microscopy

ANA was analyzed by indirect IF microscopy using multispot slides with fixed HEp-2 cells (ImmunoConcepts, Sacramento, CA, USA) as antigen substrate and fluorescein-isothiocyanate (FITC) conjugated gamma-chain specific anti-human IgG as detection antibody (DAKO, Glostrup, Denmark). The cut-off level for a positive ANA test was set at a titer of 1:200, corresponding to >95th percentile among 150 healthy female blood donors. Positive ANA tests were categorized regarding IF staining patterns (H-ANA, S-ANA, HS-ANA, N-ANA ± other pattern, or other staining patterns [here designated oANA]). To qualify as an H-ANA pattern, chromatin staining was required in metaphase/anaphase cells and, likewise, absence of chromatin staining was required to qualify as a pure S-ANA pattern. Microscope slides with fixed *Crithidia luciliae* (ImmunoConcepts) and FITC conjugated gamma-chain specific anti-human IgG (DAKO) were used to analyze IgG-class anti-dsDNA antibodies by IF with a cut-off titer at 1:10, corresponding to >99th percentile among 100 (50 males/50 females) healthy blood donors.

Anti-ENA antibodies

Autoantibodies to ENA included the following specificities: Ro/SSA, La/SSB, Sm, snRNP, Scl-70 and Jo-1, and were analyzed by DRID (ImmunoConcepts) and/or line-blot technique (ProfilePlus, R052 Euroassay, Euroimmun, Lübeck, Germany). In the case line-blot screening resulted in positive reactions regarding antibodies against Sm, Jo-1 or Scl-70, these specificities were confirmed by DRID in order to qualify as positive. For the other anti-ENA specificities, good reproducibility has been reassured at the performing laboratory.

Routine laboratory analyses

To assess hematologic and renal disorders, laboratory tests at selected visits included hemoglobin and blood cell counts (erythrocytes, total leukocyte count, lymphocytes, neutrophils and platelets) as well as urinalysis (dip-slide procedure for erythrocytes, protein and glucose), urinary sediment assessment and serum creatinine. Lupus anticoagulant was performed by the dilute Russell's viper venom test (DRVVT).

Renal histopathology

38 of the included patients (*i.e.* 79% of those who fulfilled ACR-82 criterion number 7 'renal disorder') had undergone renal biopsy performed by percutaneous ultrasonography-guided puncture in accordance with a standard protocol. The renal tissue obtained was classified according to the WHO classification for lupus nephritis [39]. All biopsies were evaluated by conventional light microscopy, direct IF and electron microscopy.

Statistics

Frequencies of the different IF-ANA staining patterns in the study group were analyzed to identify subgroups for further analyses. Clinical and laboratory features were described by their frequencies, for each of most common pattern subgroups separately. Differences in distributions of different staining patterns regarding clinical and laboratory features were analyzed using Chi-square tests of independence (alternatively Fisher's exact test in case of small expected frequencies) with Cramer's V as measure of effect size. All statistics were performed using IBM SPSS 20.0. For each statistical test, exact *p*-values (non-adjusted) are reported.

Ethical considerations

Oral and written informed consent was obtained from all subjects. The study protocol was approved by the regional ethics committee in Linköping, Sweden (M75-08/2008).

RESULTS

Frequencies of clinical and laboratory features are displayed in Table 1ab. 219 of 222 (99%) were found to be ever ANA positive. Skin disease and arthritis were the most commonly fulfilled ACR-82 criteria followed by 'hematologic disorder'. 22% of the patients had renal disease and 44% showed positive anti-dsDNA antibody test at least once during their disease course. However, 5 individuals were classified with unknown or other pattern (oANA) since the Clinical immunology laboratory was unable to recover documentation of IF-ANA patterns or classified the positive nuclear staining pattern as very rare (nuclear dots). 4 of these 5 individuals were prescribed at least one disease-modifying drug. H-ANA staining was by far the most frequent pattern (54%) followed by S-ANA (22%), HS-ANA (11%), N-ANA ± other pattern (9%) and C-ANA (1%). The first 4 pattern groups were considered large enough for statistical comparisons.

Some clinical and laboratory features showed differences in proportions over different staining patterns (Table 1). 'Immunologic disorder' (the 10th ACR-82 criterion) and anti-dsDNA antibodies were more often associated with H-ANA, and less often associated with S-ANA; whereas anti-snRNP showed the opposite direction (moderate to strong effects). Central nervous system disease was less often associated with H-ANA compared to other staining patterns, but the number of affected individuals was very low. Anti-Sm was more often, whereas arthritis and organ damage (SDI ≥1) respectively were less often, associated with S-ANA. Anti-Ro/SSA and anti-La/SSB antibodies were more often associated with HS-ANA. No significant differences in proportions of the number of concomitant anti-nuclear antibody fine-specificities over different staining patterns

were recorded.

Table 1a

Clinical feature (ACR–82)	H-ANA (%)	S-ANA (%)	HS-ANA (%)	N-ANA* (%)	*P*-value	Cramer's V	C-ANA (%)	oANA (%)	Total (%)
	(*n* = 119)	(*n* = 49)	(*n* = 24)	(*n* = 19)			(*n* = 3)	(*n* = 5)	
Malar rash	42.0	53.1	41.7	31.6	0.38		0	80	43.8
Discoid lupus	12.6	18.4	20.8	10.5	0.57†		33	20	15.1
Photosensitivity	47.9	65.3	58.3	36.8	0.09		33	80	52.5
Oral ulcers	10.1	16.3	12.5	10.5	0.68†		0	0	11.4
Arthritis	76.5	63.3 –	91.7	89.5	0.02	0.23	100	100	77.2
Serositis	42.9	38.8	25.0	47.4	0.38		100	20	40.6
Pleuritis	38.7	34.7	25.0	36.8	0.64		100	20	36.5
Pericarditis	16.0	14.3	0.0	15.8	0.15†		33	0	13.7
Renal disorder	24.4	16.3	29.2	15.8	0.49		33	0	21.9
Neurologic disorder	1.7 –	8.2	8.3	10.5	0.04†	0.17	33	0	5.0
Seizures	0.8 –	6.1	8.3	10.5	0.02†	0.19	33	0	4.1
Psychosis	0.8	2.0	0.0	5.3	0.22†		0	0	1.4
Hematologic disorder	48.7	59.2	58.3	42.1	0.45		33	0	50.2
Immunologic disorder	64.7 +	24.5 –	33.3	31.6	<0.001	0.37	33	0	47.5
Antinuclear antibody	100	100	100	100			100	100	100
≥ 6 fulfilled ACR criteria	26.9	24.5	20.8	15.8	0.73		33	0	24.2
SDI score ≥ 1	59.7	30.6 –	54.2	57.9	0.007	0.24	67	60	52.5

H = Homogenous. S = Speckled, HS = Homogenous/Speckled, N = Nucleolar, C = Centromeric, oANA = other pattern, + = positive association, – = negative association.

* Staining pattern ± combination with other pattern(s).

† Fisher's exact test.

Table 1b

Laboratory feature	H-ANA (%) (*n* = 119)	S-ANA (%) (*n* = 49)	HS-ANA (%) (*n* = 24)	N-ANA* (%) (*n* = 19)	*P*-value	Cramer's V	C-ANA (%) (*n* = 3)	oANA (%) (*n* = 5)	Total (%)
Hemolytic anemia	2.5	8.2	4.2	5.3	0.30†		0	0	4.1
Leukocytopenia	29.4	30.6	33.3	21.1	0.84		33	0	28.8
Lymphocytopenia	27.7	32.7	33.3	31.6	0.90		0	0	28.8
Thrombocytopenia	10.1	16.3	12.5	5.3	0.59†		0	0	11.0
Lupus anticoagulant#	34.6	24.3	33.3	38.5	0.68		33	50	32.5
Anti-dsDNA	63.9 +	12.2 –	33.3	26.3	<0.001	0.45	33	0	43.8
Anti-Sm	3.4	16.7 +	4.2	10.5	0.022†	0.21	0	0	7.0
Anti-Ro/SSA	32.8	43.8	62.5 +	36.8	0.047	0.20	33	0	38.5
Anti-La/SSB	7.0	12.8	33.3 +	0	0.002†	0.29	0	0	11.8
Anti-snRNP	6.9 –	47.8 +	13.6	22.2	<0.001†	0.43	0	0	20.2

H = Homogenous. S = Speckled, HS = Homogenous/Speckled, N = Nucleolar, C = Centromeric, oANA = other pattern, + = positive association, – = negative association.

* Staining pattern ± combination with other pattern(s).

† Fisher's exact test.

Not analyzed in all patients; H-ANA: *n* = 81, S-ANA: *n* = 37, HS-ANA: *n* = 18, N-ANA: *n* = 13, C-ANA: *n* = 3, oANA: *n* = 2.

Photosensitivity was significantly associated with anti-Ro/SSA antibodies (Figure 2). On the contrary, arthritis was less common among patients with anti-Ro/SSA antibodies. A positive anti-Sm antibody test was significantly associated with lymphocytopenia (Fisher's exact test, p =0.014, Cramer's V =0.19); and as expected, a positive anti-dsDNA antibody test was significantly associated with renal disorder (Chi-square test, p <0.001, Cramer's V =0.34).

The proportions of different staining patterns in the group of patients fulfilling only the Fries' criteria and those meeting the ACR-82 criteria are demonstrated in Figure 3. The higher proportion of patients with nucleolar staining in the Fries' group as compared to the ACR-82 group did not meet statistical significance (Fisher's exact test, p =0.064). Figure 4 demonstrates the number of fulfilled ACR criteria in relation to nuclear staining patterns. H-ANA was found to dominate regardless of the number of fulfilled ACR criteria. As indicated in Figure 5, H-ANA was significantly more common in patients that had been classified with proliferative lupus nephritis (WHO class 3 or 4) on renal biopsy (Chi-square test, p <0.001) compared to other staining patterns.

DISCUSSION

The use of IF microscopy to identify antinuclear antibodies was introduced by Holman, Kunkel and Friou already in the early 1950's [40, 41], and still remains the gold standard for ANA diagnostics [9, 42]. Different IF-ANA staining patterns arise depending on the nuclear antigens targeted and, to some extent, the nuclear staining patterns can have diagnostic implications [10, 13]. Being an exceptionally heterogeneous disease entity, different SLE phenotypes may associate with different ANA subspecificities. Nevertheless, studies on IF-ANA staining patterns in relation to SLE subtypes are very scarce. Thus, herein we asked if the IF-ANA staining pattern of well-characterized SLE patients in a regional Swedish register *per se* contain any valuable clinical information. No corrections for multiple comparisons were made, but by reporting the exact *p*-values this can easily be done with a preferred method [43].

In a previous investigation based on South Swedish SLE patients who had all been judged IF-ANA positive at the time-point of diagnosis, a considerable proportion (24%) lost their ANA positivity over time [8]. This may appear surprising, but our findings are very consistent with the results from a recent clinical trial for belimumab in SLE [44]. Our study demonstrated that, among those remaining IF-ANA positive over time, the vast majority (62%) displayed H-ANA ± other pattern, whereas fewer had a pure S-ANA pattern (10%). In the present study, we confirmed that H-ANA is the most common IF-ANA pattern among Swedish SLE patients regardless of the number of fulfilled ACR criteria. The fact that we did not find any significant difference between ANA staining patterns in patients fulfilling the ACR-82 classification criteria and those that only met

Fries' criteria probably reflects that the ACR-82 criteria have a lower sensitivity and fail to identify all patients with 'clinical SLE' and at least 2 typical organ manifestations.

Many have dealt with differences in ANA fine-specificity and grouped patients according to ANA seroprofiles in order to reveal potential associations with defined clinical lupus manifestations [17–20, 22–24, 27–31, 45, 46]. Using the luciferase immunoprecipitation system, Ching *et al.* recently reported that the anti-Sm/snRNP-cluster was more associated with serositis than with the anti-SSA/SSB cluster [24]. Thompson and colleagues observed that SLE cases with anti-dsDNA and/or anti-Sm were more likely to have malar rash, hypocomplementemia, renal and hematologic involvement than patients without these autoantibodies [22]. Our finding of a significant association between anti-Ro/SSA and photosensitivity was expected since several studies reported that anti-Ro/SSA positive patients have an increased rate of lupus-related rash and photosensitivity [22, 27, 29]. However, in other studies the correlation between anti-Ro/SSA and skin disease has been less clear [19, 47–49].

In a recent and very large study from China, 1928 SLE patients from 5 different centers were studied according to serological profiles [50]. The presence of anti-dsDNA was found to be associated with renal disorder, serositis and hematological involvement. In our study, anti-dsDNA was exclusively associated with renal disorder. Only 15% of the Chinese lupus cohort exhibited the anti-Sm/snRNP/phospholipid-cluster, but these patients had the highest frequency of malar rash, oral ulcers, arthritis and serositis [50]. As expected, skin disease/photosensitivity was associated with the anti-SSA/SSB cluster, but contrasting to our findings Li *et al.* reported a positive association between anti-SSA

and arthritis. The reason for the contradictory findings may be sought in differences in methodology as well as in genetic factors.

Organ damage is strongly connected to SLE prognosis [51, 52], but only one biomarker (osteopontin) has so far been shown to predict organ damage [53]. In the present study, organ damage (SDI ≥1) was significantly less common among patients with S-ANA. This is a novel finding which calls for confirmation by others. A plausible explanation is that anti-dsDNA antibodies were also less common among cases with S-ANA and, given the strong association between anti-dsDNA and lupus nephritis [15, 16, 20], patients with S-ANA may have less (or at least milder) renal disease with a subsequent risk of developing organ damage. Another possible explanation is the well-documented association between anti-SSA/SSB and milder disease manifestations, *e.g.* lupus-related rash and photosensitivity [22, 27, 29]. Importantly, however, anti-SSA antibodies are not visualized on standard HEp-2 cells (used in this study) since the antigen levels are low.

To conclude, the results of this study demonstrate that IF-ANA staining patterns have some clinical correlates of potential diagnostic and prognostic interest in addition to traditional antigen-specific immunoassays. The findings that arthritis and signs of organ damage were less often associated with S-ANA compared to other staining patterns call for confirmatory studies and further elaborations, including identification of ANA fine-specificities.

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Contributors. MF was involved in conception and design of the study, data collection and manuscript writing. ÖD contributed with statistical advice, interpretation of data and drafted the paper. AK was involved in acquisition of patient data, interpretation of data, intellectual discussion and manuscript writing. TS was involved in the laboratory work, interpretation of data, intellectual discussion and drafted the paper. CS contributed to the original idea, patient characterization, interpretation of data, intellectual discussion and manuscript writing.

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Competing interests. None.

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FIGURE LEGENDS

Figure 1: Percent of SLE patients by sex and decade of age at disease onset.

Figure 2: Percentage of patients fulfilling ACR-82 criterion 3 (photosensitivity) and 5 (arthritis) in relation to anti-Ro/SSA antibody status. Photosensitivity was significantly more common, and arthritis less common, in anti-Ro/SSA antibody positive SLE patients. Data on anti-Ro/SSA antibody status was available in 216 of 222 (97.3%) cases.

Figure 3: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on those who only met the Fries' criteria and those who fulfilled at least 4 out of the 11 ACR-82 criteria.

Figure 4: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on the number of fulfilled ACR-82 criteria.

Figure 5: IF-ANA staining patterns demonstrated for the 38 patients that had undergone renal biopsy divided according to the WHO classification for lupus nephritis.

TITLE PAGE

Manuscript title: Associations between antinuclear antibody staining patterns and clinical features of systemic lupus erythematosus: analysis of a regional Swedish register

Authors: Martina Frodlund, MD[1]; Örjan Dahlström, PhD[2]; Alf Kastbom, MD PhD[1]; Thomas Skogh, MD PhD[1]; Christopher Sjöwall, MD PhD[1]*

1. Rheumatology/AIR, Department of Clinical and Experimental Medicine, Linköping University, Linköping
2. Linneaus Centre HEAD, Swedish Institute for Disability Research, Department of Behavioural Sciences and Learning, Linköping University, Linköping, Sweden

*** Corresponding author:**
Rheumatology Unit, University Hospital, SE-581 85 Linköping, Sweden
E-mail address: christopher.sjowall@liu.se
Telephone +46 10 1032416
Fax: +46 10 1031844

Running head: ANA staining patterns *versus* SLE phenotypes

Keywords: Antinuclear antibodies; Immunofluorescence microscopy; Systemic lupus erythematosus; Organ damage; Ro/SSA

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ABSTRACT

Objective. Antinuclear antibody (ANA) analysis by immunofluorescence (IF) microscopy remains a diagnostic hallmark of systemic lupus erythematosus (SLE). The clinical relevance of ANA fine-specificities in SLE has been addressed repeatedly, whereas studies on IF-ANA staining patterns in relation to disease manifestations are very scarce. This study was done to elucidate whether different staining patterns associate with distinct SLE phenotypes.

Design: Observational cohort study.

Setting: One university hospital rheumatology unit in Sweden.

Participants: The study population consisted of 222 cases (89% women; 93% Caucasians), whereof 178 met ≥4/11 of the 1982 American College of Rheumatology criteria (ACR-82). The remaining 20% had an SLE diagnosis based on positive IF-ANA (HEp-2 cells) and ≥2 typical organ manifestations at the time of diagnosis (Fries' criteria).

Outcome measures: The IF-ANA staining patterns homogenous (H-ANA), speckled (S-ANA), combined H+S (HS-ANA), centromeric (C-ANA), nucleolar±other patterns (N-ANA), and other nuclear patterns (oANA) were related to disease manifestations and laboratory measures. Antigen-specificities were also considered regarding double-stranded DNA (*Crithidia luciliae*) and the following extractable nuclear antigens: Ro/SSA, La/SSB, Sm, snRNP, Scl-70 and Jo-1 (immunodiffusion and/or line-blot technique).

Results: 54% of the SLE patients displayed H-ANA, 22% S-ANA, 11% HS-ANA, 9% N-ANA, 1% C-ANA, 2% oANA and 1% were never ANA positive. Staining patterns among patients meeting Fries' criteria alone did not differ from those fulfilling ACR-82. H-ANA was significantly associated with the 10th criterion according to ACR-82 ('immunologic disorder'). S-ANA was inversely associated with arthritis, 'immunologic disorder' and signs of organ damage.

Conclusions. H-ANA is the dominant IF-ANA pattern among Swedish SLE patients, and was found to associate with 'immunologic disorder' according to ACR-82. The second most common pattern, S-ANA, associated negatively with arthritis and organ damage.

ABSTRACT SUMMARY

Article focus:

- The use of IF microscopy to identify ANA was introduced in the early 1950's, and this technique still remains the gold standard for ANA diagnostics when screening for autoimmune diseases such as SLE.
- Different IF-ANA staining patterns arise depending on the nuclear antigens targeted and, to some extent, the nuclear staining patterns can have diagnostic implications.
- Herein, we asked if the IF-ANA staining pattern of well-characterized SLE patients included in a regional Swedish register contain any valuable clinical information regarding distinct SLE phenotypes.

Key messages:

- Regardless of the number of fulfilled ACR-82 criteria, H-ANA was the most common IF-ANA pattern among Swedish SLE patients; and this staining was strongly associated with 'immunologic disorder' and anti-dsDNA antibodies.
- S-ANA was the second most common pattern and associated negatively with arthritis and organ damage. These intriguing findings are novel and need to be replicated.
- As previously shown by others, photosensitivity was significantly associated with anti-Ro/SSA antibodies. On the contrary, arthritis was less common among patients with anti-Ro/SSA antibodies.

Strengths and limitations of this study:

- The large study population with thoroughly organized data and very few internal missing values constitute the strength of this study.

- Although this study confirmed several known associations between serological findings and clinical features, it did not have the power to allow comparisons with specific types of cutaneous lupus, renal disease, central or peripheral nervous system manifestations, as well as with clinical features not included in the ACR criteria.

INTRODUCTION

The clinical spectrum of systemic lupus erythematosus (SLE) is exceedingly variable with an unpredictable disease course characteristically with episodes of flares and remissions. Ongoing disease exacerbations and cumulative damage/dysfunction over time can significantly interfere with quality of life [1]. Organ systems most commonly involved in SLE include joints, skin, mucous membranes, bone marrow, and kidneys. Despite the considerable differences between SLE patients, the occurrence of antinuclear antibodies (ANA) in serum at the time of diagnosis is a common finding with very few exceptions [2].

An "abnormal titer" of ANA assessed by immunofluorescence (IF) microscopy (IF-ANA) is one of the 11 criteria for SLE according to the 1982 American College of Rheumatology (ACR-82) validated classification criteria [3] as well as the 1997 revised criteria [4]. Also the recently proposed Systemic Lupus International Collaborating Clinics (SLICC) criteria state that an ANA test "above the laboratory reference value" remains a criterion for SLE, but without specifying the method for ANA assessment [5]. Unfortunately, none of the classification grounds state how to define the cut-off level for ANA. Similar to the definition of a positive rheumatoid factor test according to the 1987 ACR classification criteria for rheumatoid arthritis (RA) [6], we advocate a cut-off level of >95th percentile among healthy female blood donors to define an abnormal level of ANA analyzed by indirect IF microscopy utilizing fixed HEp-2 cells as source of nuclear antigens and, importantly, gamma-chain specific secondary antibodies to pinpoint IgG-class IF-ANA [7]. At this cut-off level, ANA has very high diagnostic sensitivity for SLE, but low diagnostic specificity, with close to 5% prevalence among healthy female blood

donors [8]. Accordingly, ANA testing should only be done upon fair clinical indications of ANA-related disease. Although circulating levels of ANA may vary over time in SLE patients, the correlation between IF-ANA titer and clinical activity is poor [9].

Nuclear constituents such as histone proteins, double-stranded (ds) DNA, DNA/histone complexes (nucleosomes), various nuclear enzymes and other proteins/ribonucleoproteins are common target antigens for ANA. On the basis of their different intra-nuclear distributions, IF-ANA staining patterns can be subdivided into homogenous /chromosomal (H-ANA), centromeric (C-ANA), speckled/extrachromosomal (S-ANA), nucleolar (N-ANA), nuclear membrane, nuclear dot and other defined patterns [10]. The most common ANA pattern detected among healthy individuals has been reported as a uniformly distributed staining of HEp-2 cells in the interphase and a chromosomal staining in dividing cells, designated 'dense fine speckled pattern', whereas we have actually referred to this staining as a homogenous/chromosomal pattern, *i.e.* H-ANA [8, 11, 12]. A 'classical' homogenous/chromosomal pattern is the most common among SLE patients from southern Sweden as well as in RA [8]. Antibodies against dsDNA, histones and DNA/histone complex all yield a 'classical' H-ANA pattern on HEp-2 cells [13]. Antibodies against dsDNA, histones and DNA/histone complex all yield an H-ANA pattern [13]. The presence of anti-dsDNA, which is included in ACR-82 criterion number 10 designated 'immunologic disorder', has been regarded as a fairly specific diagnostic marker of SLE and is very common in lupus nephritis [2, 10, 13–15]. S-ANA is generated by antibodies targeting 'extractable nuclear antigens' (ENA), *i.e.* a group of extra-chromosomal antigens which are readily extracted with 0.15M sodium chloride, for instance 'small nuclear ribonucleoprotein' (snRNP) and the 'Smith antigen' (Sm), which

are both located on U1-RNP particles [2, 10, 13]. Anti-Sm antibody detected by double radial immunodiffusion (DRID) in gel is highly specific for SLE and practically always occurs together with anti-snRNP. Anti-Sm has been reported to associate with constitutional symptoms (pyrexia, weight-loss and fatigue), nephritis and central nervous system disease, but the sensitivity in cohorts worldwide varies dramatically due to ethnicity [13, 17–21]. Anti-Sm has also been reported to associate with serositis and Raynaud's phenomenon [19, 22–24]. N-ANA patterns are not typical of SLE, but rather of systemic sclerosis of the diffuse type [25]. N-ANA may be directed against *e.g.* fibrillarin, RNA-polymerase 1–3, 'PM-Scl', and Scl-70 (topoisomerase-1) [10, 13, 26]. Scl-70, which belongs to the 'ENA family', is also found both extra-chromosomally in the nucleoplasm and bound to DNA, thus giving rise to a mixed IF staining pattern. Like Scl-70, the La/SSB antigen may partly localize in nucleoli. Most experience regarding clinical associations to anti-ENA refers to DRID analyses. As regards anti-La/SSB as well as anti-Ro/SSA, a positive DRID test is clinically linked to Sjögren's syndrome and to some extent SLE [22, 27–31]. A positive anti-La/SSB DRID test generally occurs together with anti-Ro/SSA, whereas anti-Ro/SSA is frequently demonstrated in the absence of anti-La/SSB. Since the concentration of Ro/SSA is low in HEp-2 cells, anti-Ro/SSA escapes detection when non-transfected HEp-2 cells are used as ANA substrate for IF microscopy [32]. In a small proportion of pregnant women with circulating anti-Ro52/SSA, transplacental antibody passage to the fetus can result in neonatal lupus, *i.e.* typical congenital skin rash (which vanishes in parallel with elimination of the maternal antibodies) and sometimes also in congenital lifelong complete atrioventricular heart block [33, 34].

Although several studies have dealt with the clinical significance of ANA fine-specificities in SLE, very few have evaluated if/how different IF-ANA staining patterns may relate to distinct clinical lupus features. In the present study we aimed at comparing IF-ANA staining patterns with defined clinical and laboratory disease manifestations among well-characterized cases of SLE.

PATIENTS AND METHODS

Subjects

222 SLE patients (198 women and 24 men; mean age 51 years; range 18–88) taking part in the prospective follow-up programme KLURING (a Swedish acronym for 'Clinical LUpus Register in Northeastern Gothia') at the Rheumatology clinic, Linköping university hospital, Sweden were included between September 2008 and November 2012. This corresponds to about 95% of the expected SLE cases in the catchment area of Linköping and ≥98% of all known SLE cases. The patient material was recently described in detail [35]. 178 patients (80%) met the ACR-82 criteria [3], and 44 (20%) had a clinical diagnosis of SLE based on a history of abnormal ANA titer (specified below), and at least 2 typical organ manifestations at the time of diagnosis (referred to as the Fries' criteria) [36, 37]. The presence of anti-cardiolipin antibodies of IgG and/or IgM class detected by ELISA and/or positive lupus anticoagulant test (not classified as an immunologic criterion according to ACR-82) was found in 31 of the 44 individuals (70%) in the Fries group.

Patients were consecutively recruited; most were prevalent cases (85%), but some (15%) had newly diagnosed SLE at the time of enrollment. Distribution of age at disease onset is demonstrated in Figure 1. The median disease duration by year 2012 was 12 years (mean 13.4; range 0–49). Disease severity/organ damage was estimated using the SLICC/ACR damage index (SDI) at the end of year 2011 or from the last observation made [38]. 206 (93%) of the patients were Caucasians. 92 (41%) of the patients were prescribed antimalarials (AM) alone, 68 (31%) other disease-modifying anti-rheumatic

drugs ± AM and 128 (58%) oral glucocorticoids. IF-ANA staining patterns, anti-ENA reactivity and dsDNA antibodies were analyzed on a routine basis at the Clinical immunology laboratory, Linköping university hospital and were extracted from medical records. In many patients, IF-ANA analysis was performed at several occasions over time but discrepant staining patterns were achieved in less than 5% of these cases. Herein, IF-ANA staining pattern from the time-point most adjacent to SLE onset was used for comparisons with clinical and laboratory features.

Indirect IF microscopy

ANA was analyzed by indirect IF microscopy using multispot slides with fixed HEp-2 cells (ImmunoConcepts, Sacramento, CA, USA) as antigen substrate and fluorescein-isothiocyanate (FITC) conjugated gamma-chain specific anti-human IgG as detection antibody (DAKO, Glostrup, Denmark). The cut-off level for a positive ANA test was set at a titer of 1:200, corresponding to >95th percentile among 150 healthy female blood donors. Positive ANA tests were categorized regarding IF staining patterns (H-ANA, S-ANA, HS-ANA, N-ANA ± other pattern, or other staining patterns [here designated oANA]). To qualify as an H-ANA pattern, chromatin staining was required in metaphase/anaphase cells and, likewise, absence of chromatin staining was required to qualify as a pure S-ANA pattern. Microscope slides with fixed *Crithidia luciliae* (ImmunoConcepts) and FITC conjugated gamma-chain specific anti-human IgG (DAKO) were used to analyze IgG-class anti-dsDNA antibodies by IF with a cut-off titer at 1:10, corresponding to >99th percentile among 100 (50 males/50 females) healthy blood donors.

Anti-ENA antibodies

Autoantibodies to ENA included the following specificities: Ro/SSA, La/SSB, Sm, snRNP, Scl-70 and Jo-1, and were analyzed by DRID (ImmunoConcepts) and/or line-blot technique (ProfilePlus, R052 Euroassay, Euroimmun, Lübeck, Germany). In the case line-blot screening resulted in positive reactions regarding antibodies against Sm, Jo-1 or Scl-70, these specificities were confirmed by DRID in order to qualify as positive. For the other anti-ENA specificities, good reproducibility has been reassured at the performing laboratory.

Routine laboratory analyses

To assess hematologic and renal disorders, laboratory tests at selected visits included hemoglobin and blood cell counts (erythrocytes, total leukocyte count, lymphocytes, neutrophils and platelets) as well as urinalysis (dip-slide procedure for erythrocytes, protein and glucose), urinary sediment assessment and serum creatinine. Lupus anticoagulant was performed by the dilute Russell's viper venom test (DRVVT).

Renal histopathology

38 of the included patients (*i.e.* 79% of those who fulfilled ACR-82 criterion number 7 'renal disorder') had undergone renal biopsy performed by percutaneous ultrasonography-guided puncture in accordance with a standard protocol. The renal tissue obtained was classified according to the WHO classification for lupus nephritis [39]. All biopsies were evaluated by conventional light microscopy, direct IF and electron microscopy.

Statistics

Frequencies of the different IF-ANA staining patterns in the study group were analyzed to identify subgroups for further analyses. Clinical and laboratory features were described by their frequencies, for each of most common pattern subgroups separately. Differences in distributions of different staining patterns regarding clinical and laboratory features were analyzed using Chi-square tests of independence (alternatively Fisher's exact test in case of small expected frequencies) with Cramer's V as measure of effect size. All statistics were performed using IBM SPSS 20.0. For each statistical test, exact *p*-values (non-adjusted) are reported.

Ethical considerations

Oral and written informed consent was obtained from all subjects. The study protocol was approved by the regional ethics committee in Linköping, Sweden (M75-08/2008).

RESULTS

Frequencies of clinical and laboratory features are displayed in Table 1ab. 219 of 222 (99%) were found to be ever ANA positive. Skin disease and arthritis were the most commonly fulfilled ACR-82 criteria followed by 'hematologic disorder'. 22% of the patients had renal disease and 44% showed positive anti-dsDNA antibody test at least once during their disease course. However, 5 individuals were classified with unknown or other pattern (oANA) since the Clinical immunology laboratory was unable to recover documentation of IF-ANA patterns or classified the positive nuclear staining pattern as very rare (nuclear dots). 4 of these 5 individuals were prescribed at least one disease-modifying drug. H-ANA staining was by far the most frequent pattern (54%) followed by S-ANA (22%), HS-ANA (11%), N-ANA ± other pattern (9%) and C-ANA (1%). The first 4 pattern groups were considered large enough for statistical comparisons.

Some clinical and laboratory features showed differences in proportions over different staining patterns (Table 1). 'Immunologic disorder' (the 10th ACR-82 criterion) and anti-dsDNA antibodies were more often associated with H-ANA, and less often associated with S-ANA; whereas anti-snRNP showed the opposite direction (moderate to strong effects). Central nervous system disease was less often associated with H-ANA compared to other staining patterns, but the number of affected individuals was very low. Anti-Sm was more often, whereas arthritis and organ damage (SDI ≥1) respectively were less often, associated with S-ANA. Anti-Ro/SSA and anti-La/SSB antibodies were more often associated with HS-ANA. No significant differences in proportions of the number of concomitant anti-nuclear antibody fine-specificities over different staining patterns

were recorded.

Table 1a

Clinical feature (ACR–82)	H-ANA (%) (*n* = 119)	S-ANA (%) (*n* = 49)	HS-ANA (%) (*n* = 24)	N-ANA* (%) (*n* = 19)	*P*-value	Cramer's V	C-ANA (%) (*n* = 3)	oANA (%) (*n* = 5)	Total (%)
Malar rash	42.0	53.1	41.7	31.6	0.38		0	80	43.8
Discoid lupus	12.6	18.4	20.8	10.5	0.57†		33	20	15.1
Photosensitivity	47.9	65.3	58.3	36.8	0.09		33	80	52.5
Oral ulcers	10.1	16.3	12.5	10.5	0.68†		0	0	11.4
Arthritis	76.5	63.3 –	91.7	89.5	0.02	0.23	100	100	77.2
Serositis	42.9	38.8	25.0	47.4	0.38		100	20	40.6
Pleuritis	38.7	34.7	25.0	36.8	0.64		100	20	36.5
Pericarditis	16.0	14.3	0.0	15.8	0.15†		33	0	13.7
Renal disorder	24.4	16.3	29.2	15.8	0.49		33	0	21.9
Neurologic disorder	1.7 –	8.2	8.3	10.5	0.04†	0.17	33	0	5.0
Seizures	0.8 –	6.1	8.3	10.5	0.02†	0.19	33	0	4.1
Psychosis	0.8	2.0	0.0	5.3	0.22†		0	0	1.4
Hematologic disorder	48.7	59.2	58.3	42.1	0.45		33	0	50.2
Immunologic disorder	64.7 +	24.5 –	33.3	31.6	<0.001	0.37	33	0	47.5
Antinuclear antibody	100	100	100	100			100	100	100
≥ 6 fulfilled ACR criteria	26.9	24.5	20.8	15.8	0.73		33	0	24.2
SDI score ≥ 1	59.7	30.6 –	54.2	57.9	0.007	0.24	67	60	52.5

H = Homogenous. S = Speckled, HS = Homogenous/Speckled, N = Nucleolar, C = Centromeric, oANA = other pattern, + = positive association, – = negative association.

* Staining pattern ± combination with other pattern(s).

† Fisher's exact test.

Table 1b

Laboratory feature	H-ANA (%) (*n* = 119)	S-ANA (%) (*n* = 49)	HS-ANA (%) (*n* = 24)	N-ANA* (%) (*n* = 19)	*P*-value	Cramer's V	C-ANA (%) (*n* = 3)	oANA (%) (*n* = 5)	Total (%)
Hemolytic anemia	2.5	8.2	4.2	5.3	0.30†		0	0	4.1
Leukocytopenia	29.4	30.6	33.3	21.1	0.84		33	0	28.8
Lymphocytopenia	27.7	32.7	33.3	31.6	0.90		0	0	28.8
Thrombocytopenia	10.1	16.3	12.5	5.3	0.59†		0	0	11.0
Lupus anticoagulant#	34.6	24.3	33.3	38.5	0.68		33	50	32.5
Anti-dsDNA	63.9 +	12.2 –	33.3	26.3	<0.001	0.45	33	0	43.8
Anti-Sm	3.4	16.7 +	4.2	10.5	0.022†	0.21	0	0	7.0
Anti-Ro/SSA	32.8	43.8	62.5 +	36.8	0.047	0.20	33	0	38.5
Anti-La/SSB	7.0	12.8	33.3 +	0	0.002†	0.29	0	0	11.8
Anti-snRNP	6.9 –	47.8 +	13.6	22.2	<0.001†	0.43	0	0	20.2

H = Homogenous. S = Speckled, HS = Homogenous/Speckled, N = Nucleolar, C = Centromeric, oANA = other pattern, + = positive association, – = negative association.

* Staining pattern ± combination with other pattern(s).

† Fisher's exact test.

Not analyzed in all patients; H-ANA: *n* = 81, S-ANA: *n* = 37, HS-ANA: *n* = 18, N-ANA: *n* = 13, C-ANA: *n* = 3, oANA: *n* = 2.

Photosensitivity was significantly associated with anti-Ro/SSA antibodies (Figure 2). On the contrary, arthritis was less common among patients with anti-Ro/SSA antibodies. A positive anti-Sm antibody test was significantly associated with lymphocytopenia (Fisher's exact test, p =0.014, Cramer's V =0.19); and as expected, a positive anti-dsDNA antibody test was significantly associated with renal disorder (Chi-square test, p <0.001, Cramer's V =0.34).

The proportions of different staining patterns in the group of patients fulfilling only the Fries' criteria and those meeting the ACR-82 criteria are demonstrated in Figure 3. The higher proportion of patients with nucleolar staining in the Fries' group as compared to the ACR-82 group did not meet statistical significance (Fisher's exact test, p =0.064). Figure 4 demonstrates the number of fulfilled ACR criteria in relation to nuclear staining patterns. H-ANA was found to dominate regardless of the number of fulfilled ACR criteria. As indicated in Figure 5, H-ANA was significantly more common in patients that had been classified with proliferative lupus nephritis (WHO class 3 or 4) on renal biopsy (Chi-square test, p <0.001) compared to other staining patterns.

DISCUSSION

The use of IF microscopy to identify antinuclear antibodies was introduced by Holman, Kunkel and Friou already in the early 1950's [40, 41], and still remains the gold standard for ANA diagnostics [9, 42]. Different IF-ANA staining patterns arise depending on the nuclear antigens targeted and, to some extent, the nuclear staining patterns can have diagnostic implications [10, 13]. Being an exceptionally heterogeneous disease entity, different SLE phenotypes may associate with different ANA subspecificities. Nevertheless, studies on IF-ANA staining patterns in relation to SLE subtypes are very scarce. Thus, herein we asked if the IF-ANA staining pattern of well-characterized SLE patients in a regional Swedish register *per se* contain any valuable clinical information. No corrections for multiple comparisons were made, but by reporting the exact *p*-values this can easily be done with a preferred method [43].

In a previous investigation based on South Swedish SLE patients who had all been judged IF-ANA positive at the time-point of diagnosis, a considerable proportion (24%) lost their ANA positivity over time [8]. This may appear surprising, but our findings are very consistent with the results from a recent clinical trial for belimumab in SLE [44]. Our study demonstrated that, among those remaining IF-ANA positive over time, the vast majority (62%) displayed H-ANA ± other pattern, whereas fewer had a pure S-ANA pattern (10%). In the present study, we confirmed that H-ANA is the most common IF-ANA pattern among Swedish SLE patients regardless of the number of fulfilled ACR criteria. The fact that we did not find any significant difference between ANA staining patterns in patients fulfilling the ACR-82 classification criteria and those that only met

Fries' criteria probably reflects that the ACR-82 criteria have a lower sensitivity and fail to identify all patients with 'clinical SLE' and at least 2 typical organ manifestations.

Many have dealt with differences in ANA fine-specificity and grouped patients according to ANA seroprofiles in order to reveal potential associations with defined clinical lupus manifestations [17–20, 22–24, 27–31, 45, 46]. Using the luciferase immunoprecipitation system, Ching *et al.* recently reported that the anti-Sm/snRNP-cluster was more associated with serositis than with the anti-SSA/SSB cluster [24]. Thompson and colleagues observed that SLE cases with anti-dsDNA and/or anti-Sm were more likely to have malar rash, hypocomplementemia, renal and hematologic involvement than patients without these autoantibodies [22]. Our finding of a significant association between anti-Ro/SSA and photosensitivity was expected since several studies reported that anti-Ro/SSA positive patients have an increased rate of lupus-related rash and photosensitivity [22, 27, 29]. However, in other studies the correlation between anti-Ro/SSA and skin disease has been less clear [19, 47–49].

In a recent and very large study from China, 1928 SLE patients from 5 different centers were studied according to serological profiles [50]. The presence of anti-dsDNA was found to be associated with renal disorder, serositis and hematological involvement. In our study, anti-dsDNA was exclusively associated with renal disorder. Only 15% of the Chinese lupus cohort exhibited the anti-Sm/snRNP/phospholipid-cluster, but these patients had the highest frequency of malar rash, oral ulcers, arthritis and serositis [50]. As expected, skin disease/photosensitivity was associated with the anti-SSA/SSB cluster, but contrasting to our findings Li *et al.* reported a positive association between anti-SSA

and arthritis. The reason for the contradictory findings may be sought in differences in methodology as well as in genetic factors.

Organ damage is strongly connected to SLE prognosis [51, 52], but only one biomarker (osteopontin) has so far been shown to predict organ damage [53]. In the present study, organ damage (SDI ≥1) was significantly less common among patients with S-ANA. This is a novel finding which calls for confirmation by others. A plausible explanation is that anti-dsDNA antibodies were also less common among cases with S-ANA and, given the strong association between anti-dsDNA and lupus nephritis [15, 16, 20], patients with S-ANA may have less (or at least milder) renal disease with a subsequent risk of developing organ damage. Another possible explanation is the well-documented association between anti-SSA/SSB and milder disease manifestations, *e.g.* lupus-related rash and photosensitivity [22, 27, 29]. Importantly, however, anti-SSA antibodies are not visualized on standard HEp-2 cells (used in this study) since the antigen levels are low.

To conclude, the results of this study demonstrate that IF-ANA staining patterns have some clinical correlates of potential diagnostic and prognostic interest in addition to traditional antigen-specific immunoassays. The findings that arthritis and signs of organ damage were less often associated with S-ANA compared to other staining patterns call for confirmatory studies and further elaborations, including identification of ANA fine-specificities.

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Contributors. MF was involved in conception and design of the study, data collection and manuscript writing. ÖD contributed with statistical advice, interpretation of data and drafted the paper. AK was involved in acquisition of patient data, interpretation of data, intellectual discussion and manuscript writing. TS was involved in the laboratory work, interpretation of data, intellectual discussion and drafted the paper. CS contributed to the original idea, patient characterization, interpretation of data, intellectual discussion and manuscript writing.

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Competing interests. None.

Ethics approval. The regional ethics committee in Linköping, Sweden.

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FIGURE LEGENDS

Figure 1: Percent of SLE patients by sex and decade of age at disease onset.

Figure 2: Percentage of patients fulfilling ACR-82 criterion 3 (photosensitivity) and 5 (arthritis) in relation to anti-Ro/SSA antibody status. Photosensitivity was significantly more common, and arthritis less common, in anti-Ro/SSA antibody positive SLE patients. Data on anti-Ro/SSA antibody status was available in 216 of 222 (97.3%) cases.

Figure 3: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on those who only met the Fries' criteria and those who fulfilled at least 4 out of the 11 ACR-82 criteria.

Figure 4: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on the number of fulfilled ACR-82 criteria.

Figure 5: IF-ANA staining patterns demonstrated for the 38 patients that had undergone renal biopsy divided according to the WHO classification for lupus nephritis.

Autoimmunity & Immune regulation

Department of Clinical and Experimental Medicine
AIR/Rheumatology
August 30th, 2013

To the attention of Dr. Trish Groves
Editor-in-Chief *BMJ Open*

Concerning manuscript: bmjopen-2013-003608 entitled *"Associations between anti-nuclear antibody staining patterns and clinical features of systemic lupus erythematosus: analysis of a regional Swedish register"*

Dear Dr. Groves,

Thank you for reviewing our manuscript. Please find our point-by-point responses below. We hope that you now find our manuscript acceptable for publication in *BMJ Open*.

On behalf of all authors,

Christopher Sjöwall, corresponding author

Christopher Sjöwall, *M.D. Ph.D., Associate Professor*
Rheumatology Unit, University Hospital, SE-581 85 Linköping, Sweden
christopher.sjowall@liu.se

Postal address	Telephone	Telefax	E-mail
SE- 581 85 Linköping, Sweden	+46 10 1032416	+46 10 1031844	christopher.sjowall@liu.se

Point-by-point responses to the comments made by the referees

Reviewer: Peter H Schur MD, Brigham and Womens Hospital-Harvard Med School, Medicine

1. When one is doing multiple comparisons and thus calculating p values, one needs to correct for type I error (that the differences could have been due to chance) by using a Bonferroni or equivalent correction, such as multiplying all the p values by the number of comparisons in which p values were shown to get a corrected p value--your tables show 26 p values--thus if you multiply all your p values by 26, the only noncorrected p values that will remain "significant" is a uncorrected p of <0.001

Reply: We completely agree with the reviewer that results should be associated with appropriate correction (Bonferroni is one such very conservative correction) when *interpreting* the *p*-values in Table 1a and 1b. There are many different ways to apply such a correction, which should be based on how many independent sets of tests that have been performed and then attached with a proper family-wise significance level for each such set (e.g. *Proschan MA, Waclawiw MA. Control Clin Trials 2000;21:527-39*). We had a discussion in the author-group as to whether we should decide upon a division of tests into different sets, and also on a given correction method within each such set with a given family-wise significance level. Opinions vary greatly regarding how to divide the tests and regarding the method of choice when applying the correction. According to our opinion, it is most honest to report the exact *p*-values for each test, regardless of statistical significance. We also think that the exploratory nature of this study motivates reporting the exact results of each test (hopefully they will generate other studies to confirm or reject the findings presented in this manuscript). It should thereby be an easy task for every reader, if she or he prefers, to adjust the significance level by her/his own preferred method (for example by the Bonferroni method mentioned by the reviewer). However, judged from the reviewer's comment, we realize that this was not evident from the text in the first draft of the manuscript. We have therefore included a comment that hopefully clarifies this concern:

Page 12, line 8-9:
Removed sentence: P-values <0.05 were considered significant
Replaced with: For each statistical test, exact *p*-values (non-adjusted) are reported.
Page 17, line 12-13:
Inserted sentence: No corrections for multiple comparisons were made, but by reporting the exact *p*-values this can easily be done with a preferred method [43].
Page 24:
43. Proschan MA, Waclawiw MA. Practical guidelines for multiplicity adjustment in clinical trials. *Control Clin Trials* 2000;21:527-539.

Some critical references omitted. There are a number of papers etc. omitted as follows:
1. No correlation with ANA titer and clinical activity (DH Solomon et al Arth Rheum 2002; 47:434-44

Reply: This paper was indeed already cited in the manuscript draft (ref. 9). In the revised version we have also included information on the lack of association between ANA titer and clinical SLE activity (page 6, line 2-3).

2. Specific solid phase immunoassays have largely replaced immunodiffusion tests and looking at patterns: L Cook Clin Immunol Immunopath 1998; 88:211-20

Reply: We are aware of this, but cannot see the implications for our study, where two antigen-specific methods were used: double radial immunodiffusion (DRID) and/or line blot. Contrasting to other methods, DRID identifies a subgroup of antigen-precipitating antibodies, which in some instances have higher diagnostic specificity (albeit lower sensitivity) than ELISA and other solid-phase assays. We (and other Swedish laboratories) still claim that total replacement of DRID by solid phase assays is not advisable. We advocate that some anti-ENA specificities captured by ELISA should be confirmed with 'gold standard' methods such as immunodiffusion. We and others have discussed this matter in more detail elsewhere (*Dahle C, et al. J Autoimmun 2004;22:241-48*).

Postal address	Telephone	Telefax	E-mail
SE- 581 85 Linköping, Sweden	+46 10 1032416	+46 10 1031844	christopher.sjowall@liu.se

3. Homogeneous=diffuse pattern best recognizes the DNA-histone complex (nucleosome) (and this Ab responsible for the LE cell phenomenon)
Reply: We cannot see that this remark implies additional work or revision of the manuscript. Of course, we are well aware of the fact that classical homogenous nuclear IF-staining reflects occurrence of antibodies targeting DNA-histone complexes or distinct epitopes on dsDNA or histone proteins alone, but in some cases also to other DNA-associated proteins (see also our response to point 16).

4. The peripheral (also called rim) pattern is due to anti-dsDNA, and is not seen well on Hep-2 cells, but is well seen on mouse/rat liver sections
Reply: We are also aware of the ”rim” (or rim-homogenous) hepatocyte nuclear staining pattern reflecting anti-dsDNA on cryostat sections of rat and mouse liver. However, as whole fixed HEp2 cells have replaced rat liver cryostat sections for routine purposes, we do not think that the rim pattern is a matter to discuss in the present study.

5. The Danish group have described about 20 speckled patterns--major Ab associated with speckled include anti-Sm, RNP, Ro/SSA, La/SSB, Scl-70, centromere, PCNA
Reply: Indeed, a multitude of speckled ANA patterns (as well as variants of nucleolar, nuclear dot, and nuclear membrane staining patterns) have been described. In the present study we found it adequate to concentrate on four main patterns:
(1) homogenous/nucleosomal, (2) speckled/nucleoplasmic, (3) mixed homogenous/speckled, and (4) nucleolar. In addition, we describe two small pattern groups (centromeric and ‘other ANA’) that were not considered large enough for statistical comparisons.

6. M Reichlin et al described Ro and La in SLE patients, and a few years later E Tan described SSA and SSB in patients with Sjogren's--years later they were convinced to exchange reagents, and lo and behold Ro=SSA and La=SSB--so no now we routinely say Ro/SSA and La/SSB
Reply: In accordance with the reviewer’s suggestion, we have changed the nomenclature to Ro/SSA and La/SSB all through the manuscript.

7. For a ACR sponsored review of the significance of anti-Sm and anti-RNP by immunodiffusion and ELISA (EIA) see: Arth & Rheum 2004; 51:1030-44--which gives better data than cited by the authors
Reply: This paper has been added to the reference list.

8. For the ACR sponsored review of anti-DNA see ARTH RHEUM 2002; 47:546-555
Reply: This paper has been added to the reference list.

9. Solid phase assays (ELISA, EIA) have virtually replaced immunodiffusion for detection of anti-DNA, ENA etc, in virtually all labs in the USA--more sensitive, easier to do, better QC, automated--see above #7 and #8
Reply: Please see our response under point 2.

10. Jill Buyon has written more recent ref that you cited#28
Reply: This reference has been replaced by a more recent publication by Dr Buyon.

11. For publication purposes we usually only include SLE patients who have 4 or more ACR criteria--having less just means they might have SLE (according to the ACR Classification criteria)
Reply: The 1982 American College of Rheumatology criteria for SLE (ACR-82) were not intended as *diagnostic* criteria, but as criteria for the *classification* of SLE. This means that the clinician may well diagnose a patient with SLE, although the ACR-82 criteria are not met. Michelle Petri and co-workers in the Systemic Lupus International Collaborating Clinics (SLICC) group recently demonstrated that the ACR-82 criteria have a low sensitivity, but a rather high specificity, for SLE (*Arthritis Rheum 2012;64:2677-86*).
The present study was done to reflect SLE in a real life clinical setting, where 20% of the SLE patients did not fulfill ≥4/11 the ACR-82 classification criteria – *NB* the presence of anti-phospholipid

Postal address SE- 581 85 Linköping, Sweden | **Telephone** +46 10 1032416 | **Telefax** +46 10 1031844 | **E-mail** christopher.sjowall@liu.se

antibodies (aPL) analyzed by ELISA or as a positive “lupus anticoagulant” test are *not* included in the “immunologic disorder” criterion according to the validated ACR-82 criteria set. Nevertheless, aPL is often applied as a classification criterion with reference to the *non-validated* update of ACR-82 suggested by Dr Hochberg in a short letter to Arthritis & Rheumatism in 1997 (*Hochberg MC. Arthritis Rheum 1997;40:1725*). In the revised version of the manuscript, we clearly state that 31 of the 44 (70%) “Fries patients” had positive aPL tests and would thus have been classified as SLE according to the “1997 update”. Nevertheless, we have refrained from applying the 1997 update, and chosen to rely on the validated ACR-82 classification criteria. In addition, we respectfully wish to stick to the “Fries’ criteria”, which we (and others, e.g. *Vikerfors A, et al. Rheumatology 2013;52:501-9*) find helpful as a “clinical check-up” for the clinical *diagnosis of SLE*.

12. ANA patterns may vary over time and at different dilutions
Reply: We completely agree with this comment. As mentioned in the *Discussion*, we found in a previous investigation based on South Swedish SLE patients who had all been judged IF-ANA positive at the time-point of diagnosis that a considerable proportion (24%) lost their ANA positivity over time (*Sjöwall C, et al. J Rheumatol 2008;35:1994-2000*). This finding is also in line with the results from a recent clinical trial for belimumab in SLE (*Wallace DJ, et al. Arthritis Rheum 2009;61:1168-78*). However, the present study had a cross-sectional design and aimed to compare the IF-ANA staining patterns from *the time-point of SLE onset* with clinical and laboratory features (see *Patients & Methods*, page 10).

13. Most USA labs use a titer of 1:40 as a cut off--SLE patients who were positive at 1:200 (or greater) who turn negative, may thus in the USA still be positive if they have a titer between 1:40 and 1:200
Reply: According to the 1982 ACR criteria, a *positive ANA test* is defined as an *abnormal* titer of ANA judged by immunofluorescence microscopy (or equivalent method). At Swedish laboratories accredited by SWEDAC for ANA diagnostics (ISO 15189), an abnormal ANA titer corresponds to ≥95th percentile among healthy female blood donors, meaning that up to 5% healthy women may be ANA-positive. The actual corresponding *cut-off titer* varies between laboratories depending on the microscope equipment including light source, filter combinations, lens, source and dilution of the fluorochrome-labelled secondary antibodies, etc! As stated in the *Methods* section, the cut-off titer (≥95th percentile) for positive IF-ANA at our laboratory was 1:200, whereas at a serum dilution of 1:40, 45% of both healthy women and men have a “positive” IF-ANA test *(Sjöwall C, et al. J Rheumatol 2008;35:1994-2000)*. Thus, a cut-off titer of 1:40 would be absurd in laboratories with modern equipment!

14. Under results you mention "immunologic disorder"--please define
Reply: In the *Results* section of the revised version of the manuscript it has been clarified that ‘Immunologic disorder’ refers to the definition of the 10th criterion according to the 1982 ACR SLE classification criteria (*Tan E, et al. Arthritis Rheum 1982;25:1271-77*). Thus, ‘immunologic disorder’ is met by at least one of the following:

a) Positive LE cell preparation
OR
b) Anti-DNA: antibody to native DNA in abnormal titer
OR
c) Anti-Sm: presence of antibody to Sm nuclear antigen
OR
d) False positive serologic test for syphilis known to be positive for at least 6 months and confirmed by *Treponema pallidum* immobilization or fluorescent treponemal antibody absorption test .

15. Results: the frequency of renal disease and anti-DNA in your cohort is lower than most other published cohorts--why--is there something unique about your group of patients--usually renal is at least 50%, and anti-DNA about 75%

Postal address SE- 581 85 Linköping, Sweden | **Telephone** +46 10 1032416 | **Telefax** +46 10 1031844 | **E-mail** christopher.sjowall@liu.se

Reply: One plausible explanation for this is that ethnicity is strongly linked to the risk of nephritis (*Hopkinson ND, et al. Ann Rheum Dis 2000;59:116-19*) with a lower frequency of renal involvement in white population. Caucasians constitute 90-95% of patients in Swedish SLE cohorts (93% in this study), and the frequencies of nephritis and anti-dsDNA antibodies do not differ much between Swedish SLE centers (unpublished data from Linköping, Stockholm, Lund, Uppsala and Umeå). For example, the percentage of newly diagnosed SLE patients with renal involvement in the late 1980's was less than 20% in southern Sweden (*Ståhl-Hallengren C, et al. J Rheumatol 2000;27:685-91*). Another likely explanation is the selection of patients. In our region, the Linköping rheumatology unit is the only clinic that diagnoses and treats SLE patients. Thus, we can conclude that about 95% of the expected SLE cases in the catchment area of Linköping and ≥98% of all known SLE cases were included in this study. Consequently, this means that our clinic takes care of the whole spectrum of SLE – from uncomplicated cases with quiescent disease and skin/joint involvement to severely ill individuals with full-blown multisystem disease.
The slightly lower frequency of anti-dsDNA antibody positive individuals observed in our cohort is probably related to the choice of method. The Clinical immunology unit at our hospital has (so far) refrained from the use of other methods for anti-dsDNA antibody detection than the *Crithidia* test (mainly because of its high specificity for SLE compared to solid-phase assays). In addition, gamma-chain specific FITC-conjugate is always used in order to only detect IgG antibodies (although not demanded by the ACR-82 criteria!).

16. Discussion: Friou not Holman and Kunkel described the IF ANA in SLE : with different cell substrates (liver, kidney, Hep-2, other cell lines) see different patterns
Reply: As a matter of fact, in 1957 Halsted Holman & Henry Kunkel published a beautiful experimental study where they described that the "LE-cell" phenomenon reflected antinuclear antibodies giving rise to a homogenous nuclear staining pattern of phagocytized ANA-opsonized leukocyte nuclei. They also showed that the autoantibodies had affinity for a nucleoprotein associated with DNA (*Holman HR, Kunkel HG. Science 1957;126:162-63*). Although not representing an orthodox means of ANA-diagnostics, it is undoubtedly an ingenious example of IF-microscopical ANA identification.

17. No association of photosensitivity with Ro:
Paz ML, Gonzalez Maglio DH, Pino M, Ferrari A, Weill FS, Nasswetter G. Leoni J. Anti-ribonucleoprotein autoantibodies in patients with systemic autoimmune diseases. Relation with cutaneous photosensitivity. Clinical Rheumatology 2011; 30:209-16.
Boey ML, Peebles CL, Tsay G, et al. Clinical and autoantibody correlations in Orientals with systemic lupus erythematosus. Ann Rheum Dis 1988; 47:918-23.
Wang CL, OOI L, Wang F: Prevalence and clinical significance of antibodies to ribonucleoproteins in systemic lupus erythematosus in Malaysia. Br Soc Rheumatol 1996, 35:2; 129-32
Mok CC, Lau CS, Chan TM, Wong RWS: Clinical characteristics and outcome of southern Chinese males with systemic lupus erythematosus. Lupus 1999,8: 188-96.
Christian N, Smikle MF, DeCeulaer K, Daniels L, Walravens MJ, Barton EN. Antinuclear antibodies and HLA class II alleles in Jamaican patients with systemic lupus erythematosus. West Indian Med J 2007; 56: 130-3.
Reply: We agree with the reviewer that this question may be controversial. However, when we scrutinized these papers lack of association between photosensitivity and anti-Ro/SSA was evident only in 3 cases (*Paz ML, et al. Clin Rheumatol 2011; Boey ML, et al. Ann Rheum Dis 1988; Wang CL, et al. Br J Rheumatol 1996*). Thus, these papers were included in the reference list along with the following paper: *Sutej PG, et al. Photosensitivity and anti-Ro (SS-A) antibodies in black patients with systemic lupus erythematosus (SLE). Br J Rheumatol 1989;28:321-324.*

Reviewer: Catharina Eriksson MD, Department of Clinical Immunology/Clinical Microbiology, Umeå University, S-901 87 Umeå, Sweden.

1) Page 8, Patients and methods: The authors describe their definition of cut-off level for IF-ANA as >95th percentile for female blood donors, and conclude that this has a very high sensitivity for SLE.

Postal address	Telephone	Telefax	E-mail
SE- 581 85 Linköping, Sweden	+46 10 1032416	+46 10 1031844	christopher.sjowall@liu.se

There are indeed 24 men with SLE in the study. The authors have to better describe the cut-off level for and the sensitivity for SLE by the described criteria even for the male patients.
Reply: This remark is well taken. However, as mentioned in *Patients & Methods* (page 10) "*IF-ANA staining patterns, anti-ENA reactivity and dsDNA antibodies were analyzed on a routine basis at the Clinical immunology laboratory, Linköping university hospital and were extracted from medical records. In many patients IF-ANA analysis was performed at several occasions over time, but discrepant staining patterns were achieved in less than 5% of these cases. Herein, IF-ANA staining pattern from the time-point most adjacent to SLE onset was used for comparisons with clinical and laboratory features*". Although there is indeed support for the use of different cut-offs for men and women, it is not realistic to perform this on a routine basis. In addition, the vast majority of the SLE cases had ANA titers considerably above 1:200 including all of the 24 men. All three of the ANA negative cases were women.

2) Page 9, Indirect IF microscopy: Line 38 - was the secondary antibody also for anti-dsDNA FITC conjugated gamma-chain specific anti-human IgG? Was the cut-off level for anti-dsDNA also referring to female blood donors?
Reply: The secondary FITC-conjugated antibody used in the anti-dsDNA antibody assay was indeed also gamma-chain specific (information now provided in the revised version). The cut-off level for anti-dsDNA referred to 50 female and 50 male healthy blood donors (none of them anti-dsDNA antibody positive).

3) Page 9, Anti-ENA antibodies: SSA should be defined with respect to Ro-60 and Ro-52, and if possible report these separately.
Reply: Since this study is based on historical data extracted from medical records (see reply to first comment) from the time-point of SLE onset, complete anti-ENA antibody profiles were unfortunately not available in a majority of the patients.

4) Routine laboratory analyses, page 10: Lupus anticoagulant was analysed, but not anti-cardiolipin antibodies. Why? A comment should be added.
Reply: Again, the study is based on historical IF-ANA data from the time-point of SLE onset. While the lupus anticoagulant test (dilute Russell's viper venom test) has been very stable over the years, this has unfortunately not been the case with the anti-cardiolipin antibody assay. In fact, since the 1990's several different assays with different cut-offs have been used at the Clinical immunology unit. Samples that previously were judged as low anti-cardiolipin antibody positive are now negative with the present automated method. Thus, good reproducibility was not reassured with the anti-cardiolipin assay and this is the reason why we chose to exclude these data.

1) A couple of more questions should be of interest to know: did the disease duration, or the number of nuclear antibodies have any impact on the clinical feature?

Reply: Since this study is based on historical autoantibody data from the time-point of SLE onset, it does not make sense to compare the antibody patterns/nuclear antibodies with disease duration. However, as suggested by the reviewer, we made additional analyses of the *number of nuclear antibodies* in comparison with IF-ANA patterns (shown in table below). The maximum number of concommitant anti-nuclear antibody fine-specificities recorded was 5 (anti-dsDNA, anti-Sm, anti-Ro/SSA, Anti-La/SSB and anti-snRNP).

	0	**1**	**2**	**3**	**4**
H	32	52	24	11	0
S	11	21	11	4	2
N	8	7	2	1	1
H/S	6	6	7	5	0

Patients with 2, 3 or 4 ANA-fine-specificities (none had 5!) were put in one group (three groups were composed; 0, 1 or >1 nuclear antibodies) and proportions of different staining patterns were tested. There were, however, no significant differences in proportions of the number of antibodies over dif-

Postal address SE- 581 85 Linköping, Sweden | **Telephone** +46 10 1032416 | **Telefax** +46 10 1031844 | **E-mail** christopher.sjowall@liu.se

ferent staining patterns (H, S, N or H/S), p=0.29. This information is provided in the revised version of the manuscript (page 13-14).

2) Table 1b: If possible - anti-SSA/Ro could be presented in Ro60 and Ro52 separately.

Reply: Since this study is based on historical data from the time-point of SLE onset, complete anti-ENA antibody profiles were unfortunately not available in a majority of the patients.

3) In the Introduction the authors refer to a study showing that homogenous ANA pattern is the most common in healthy individuals. There are indeed several publications showing that fine speckled pattern is the most common in healthy persons. The authors have to comment on this subject.

Reply: This objection is indeed relevant and well taken. The type of (very) fine-speckled pattern, which has been described in healthy individuals, also reacts with chromatin, which is evident in dividing HEp-2 cells. Therefore, we have actually labelled this pattern as "homogenous ANA". This staining pattern was not seen in the SLE material. We have now modified the text in the *Introduction* section (page 6).

Reviewer: Shinu John Ph. D, Department of Immunobiology/Laboratory Medicine, Yale University School of Medicine, USA

Were any of the patients with discrepant staining patterns excluded? The authors have adequately described the status of the patients used in the study but do not comment on exclusion criteria, if any

Reply: All SLE patients within the county of Östergötland meeting either the 1982 American College of Rheumatology criteria for SLE (ACR-82) or the "Fries' criteria" (defined in *Patients & Methods*) were offered to take part of this structured follow-up programme. Less than 5% of suitable patients have denied participation for different reasons. The ethical permission did not allow us to use data from patients that refrained from participation in the follow-up programme. No other exclusion criteria were used.

Do the various drug treatments alter the ANA patterns? Since a significant number of patients were on some sort of disease modifying drug, the table should indicate the category of drug and the predominant ANA pattern.

Reply: To answer the question raised by the reviewer, one would need a longitudinal study. The present study had a cross-sectional design and aimed to compare the IF-ANA staining patterns from the *time-point of SLE onset* with clinical and laboratory features. Consequently, the vast majority of patients did not have any disease-modifying drug (but possibly glucocorticoids) when the ANA test was performed. Provided data on disease-modifying drugs refer to what the patients were prescribed once the SLE diagnosis was confirmed.

Our experience, however, is that a considerable proportion (24%) of SLE patients lose their ANA positivity over time (*Sjöwall C, et al. J Rheumatol 2008;35:1994-2000*). In the present study, patients that remained ANA positive over time mainly displayed a constant pattern: "*In many patients, IF-ANA analysis was performed at several occasions over time but discrepant staining patterns were achieved in less than 5% of these cases*" (page 10).

Where mixed ANA patterns are observed, sera should be further diluted and IF assays performed to assess the most dominant pattern

Reply: As mentioned in *Patients & Methods* (page 10) "*IF-ANA staining patterns, anti-ENA reactivity and dsDNA antibodies were analyzed on a routine basis at the Clinical immunology laboratory, Linköping university hospital and were extracted from medical records."* Since further dilution of serum samples with mixed ANA patterns is not part of the clinical routine at our Clinical immunology laboratory, we have no possibilities to perform this.

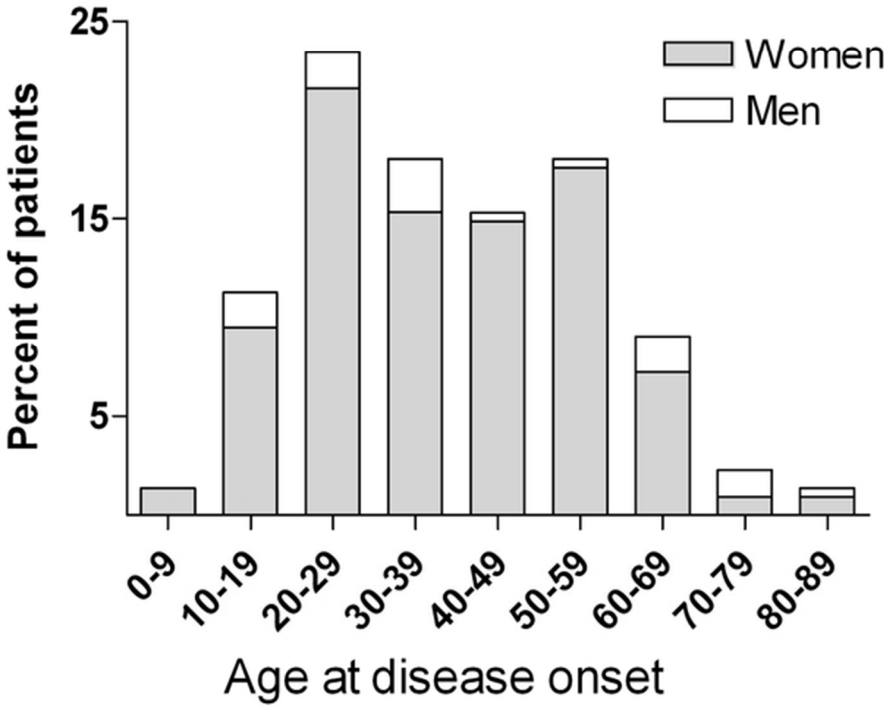
Please include representative IF images of other ANA (oANA) pattern. Were these patients on any "disease modifying drug"?

Postal address SE- 581 85 Linköping, Sweden | **Telephone** +46 10 1032416 | **Telefax** +46 10 1031844 | **E-mail** christopher.sjowall@liu.se

Reply: The 5 SLE patients in the "oANA group" consisted of 1 case with a pattern very rarely seen in SLE patients (nuclear dots) and in 4 cases the Clinical immunology laboratory was unable to recover documentation of the IF-ANA patterns, but had classified the samples as "*IF-ANA positive*". 4 of these 5 individuals were prescribed at least one disease-modifying drug. This information is now given in the text (page 13).

It would be helpful to have RF and Blys/BAFF titers of these patients
Reply: Unfortunately, neither RF nor Blys/BAFF levels were routinely measured at inclusion in our cohort. We are not familiar with whether or not RF and Blys/BAFF levels are associated with certain SLE phenotypes or specific IF-ANA patterns.

Postal address SE- 581 85 Linköping, Sweden
Telephone +46 10 1032416
Telefax +46 10 1031844
E-mail christopher.sjowall@liu.se



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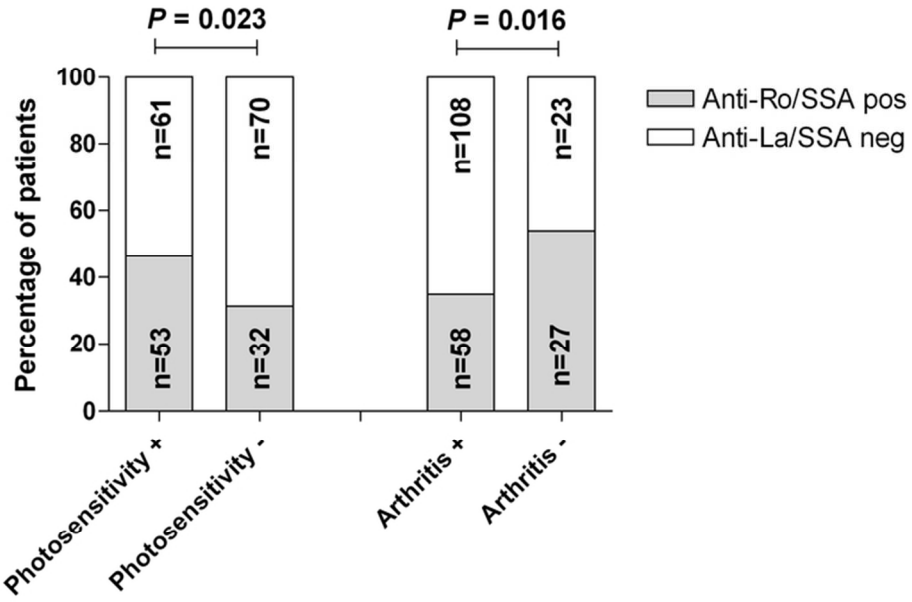

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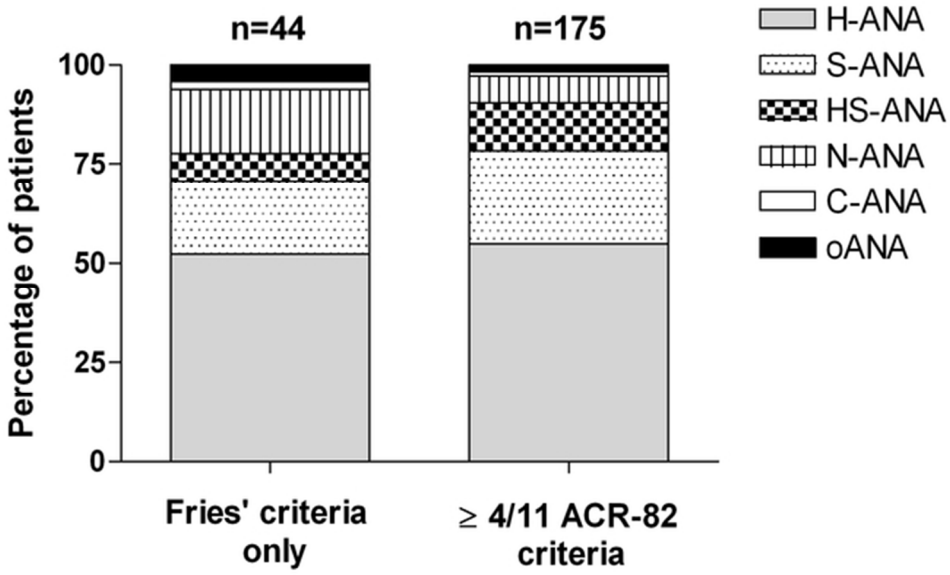

Figure 3: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on those who only met the Fries' criteria and those who fulfilled at least 4 out of the 11 ACR-82 criteria.
57x36mm (300 x 300 DPI)

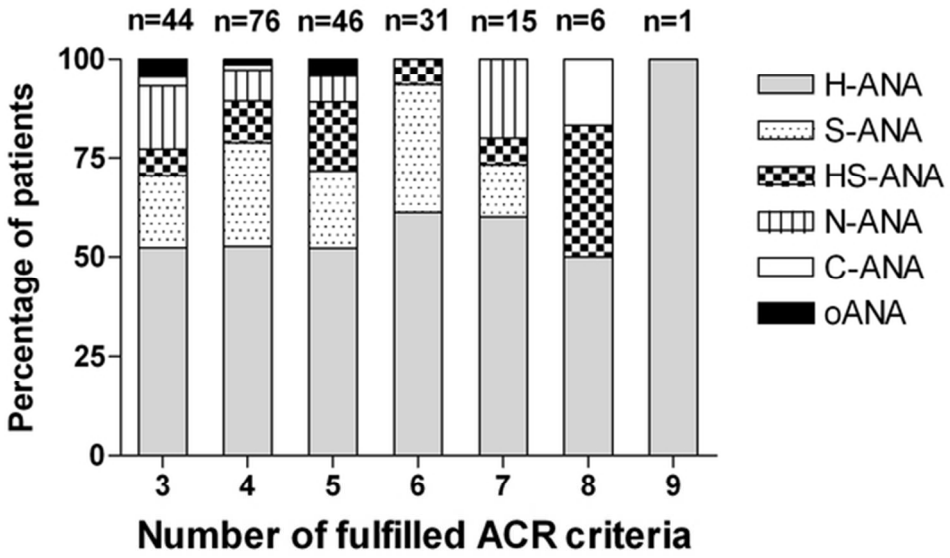

Figure 4: IF-ANA staining patterns demonstrated for the 219 ever ANA positive SLE patients divided on the number of fulfilled ACR-82 criteria.
56x34mm (300 x 300 DPI)

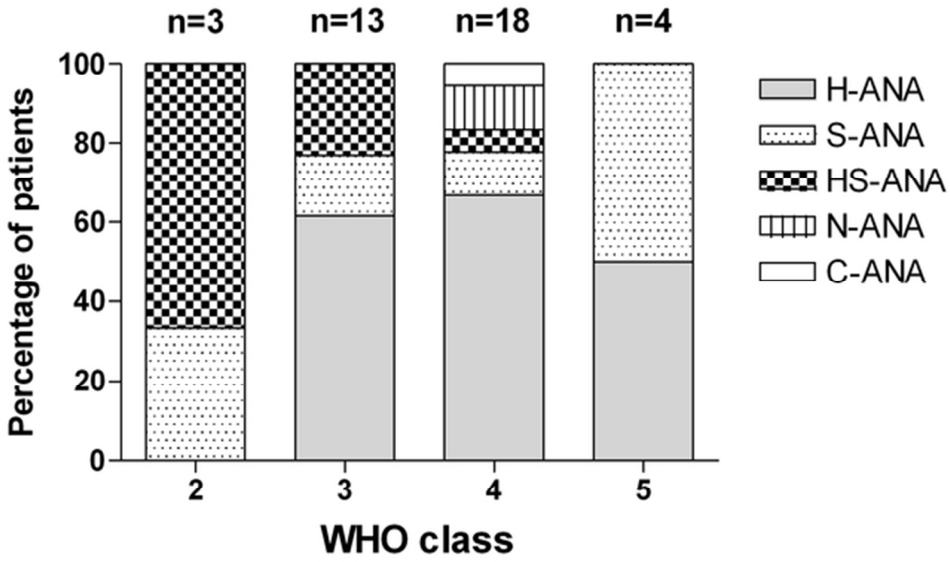

Figure 5: IF-ANA staining patterns demonstrated for the 38 patients that had undergone renal biopsy divided according to the WHO classification for lupus nephritis.
57x35mm (300 x 300 DPI)