Curr Treat Options in Rheum (2019) 5:147–167
DOI 10.1007/s40674-019-00122-0

Lupus (S Keeling, Section Editor)

Challenges and Advances in SLE Autoantibody Detection and Interpretation

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Published online: 7 May 2019

This article is part of the Topical Collection on *Lupus*

Keywords SLE · Antinuclear antibodies (ANA) · Review · Autoantibodies

Abstract

Purpose of review This review is an overview of key autoantibodies used in the diagnosis and management of SLE. Questions addressed are the advantages to but limitations of ANA testing and what are the key considerations in ordering ANA tests and then interpreting the ANA results.
Recent findings There is a progressive move towards
- Solid-phase multi-analyte arrays with algorithmic analysis (SPMAAA)
- Closing the seronegative gap in SLE
- Harmonization of ANA testing

Summary As an approach to limiting morbidity and rising health care costs associated with SLE, the future of ANA testing should focus on making an accurate and actionable diagnosis of very early SLE. To achieve this goal, harmonization of autoantibody testing will be important.

Introduction

More than a half century has lapsed since the serendipitous discovery of the lupus erythematosus (LE) cell in systemic lupus erythematosus (SLE) and the development of the LE cell test by Hargraves and his colleagues at the Mayo Clinic [1]. While the description of cardiolipin and the biological false-positive Venereal Diseases Research Laboratory (VDRL) test in SLE antedated the LE cell (reviewed in [2]), the LE cell discovery still serves as a historic reference point for the earliest studies of antinuclear antibodies (ANA). In the following 70 years, the spectrum of clinicians that use ANA for diagnosis and decision-making has markedly widened [3, 4]. For

example, ANA testing was once regarded the exclusive domain of rheumatologists and clinical immunologists, but today, it is also used by primary care providers and virtually all other subspecialists including nephrologists, dermatologists, respirologists, and neurologists [4]. The primary driver of this change is the remarkable spectrum of autoantibodies reported in systemic autoimmune rheumatic disease (SARD) and other autoinflammatory syndromes; in SLE alone, more than 180 autoantibodies have been described [5, 6]. Some might regard the continuing search for new "esoteric" or rare autoantibodies in SLE [7] unnecessary but, for one thing, since approximately 5% of SLE patients are "seronegative" [8•], at a minimum, these efforts continue to narrow that "seronegative gap" [9].

There has also been a remarkable transition in the technologies used to detect autoantibodies that has been fostered by the "Golden Age" of cell and molecular biology that emerged in the mid-1970s [3, 10, 11•]. While some of the earlier immunoassays such as the LE cell test, double immunodiffusion, hemagglutination, complement fixation, radioimmunoassays, and counterimmunoelectrophoresis are rarely used today [10], the contemporary ANA indirect immunofluorescence assay (IFA) on HEp-2 cell substrates is increasingly used and has also become the screening test of choice for SLE [12]. One innovation that has sustained the use of ANA IFA's is automated digitized microscopy that is capable of pattern recognition and, in some devices, digital algorithms to perform single-well titers [3, 13]. The American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) are about to release new criteria (reviewed in [14, 15]) based on a scoring system including a positive ANA at a titer ≥1:80 by IFA ("or its equivalent") occurring at least once as an entry criterion [16].

Newer solid-phase multi-analyte arrays (SPMAA) [17] have also emerged with higher throughput, sensitivity, and specificity while detecting a broader range of autoantibodies in comparatively miniscule serum samples [18]. A technological gap, however, is that SPMAA does not have on-board, real-time standards or calibrators for each antigen in an array, a gap that can be overcome [9]. Recent evidence also suggests that SPMAA is a more cost-effective approach than ANA IFA [19, 20]. Universally accepted, standardized, and cost-effective follow-up ANA testing algorithms for SLE need to be developed. And with the advent of a new SLE classification criteria, better technologies, and novel autoantibodies, the purposes of this review are to summarize the advantages to and limitations of current ANA testing and to provide a general overview of important autoantibodies in SLE.

Important considerations in interpretation of ANA test results and reports

Before considering the clinical value of SLE-specific and SLE-associated autoantibodies, clinicians need to be aware of important limitations to ANA testing and interpretation of ANA test results (Table 1). Unfortunately, many multicenter studies and metanalysis seemingly fail to recognize the marked variability of ANA testing across different jurisdictions and how that impacts on the conclusions of their studies [21, 22]. Autoantibodies are extremely complex because within any individual they can vary with time (although the precise chorological dynamics are poorly understood) and also vary between patients and even within a patient over the clinical course of SLE [23••, 24]. This variability is likely related to the highly differing post-diagnostic clinical course of SLE that may be "relapsing-remitting," "chronically active," or "prolonged quiescence" [25]. In addition, the impact of therapeutic interventions and coincidental co-morbidities is very difficult to control for when results are obtained from cross-sectional samples. Hence, a critical factor in interpretation of ANA test is to appreciate that results obtained from cross-sectional SLE cohorts can be markedly different from inception cohorts because single-point

Table 1. Key considerations when interpreting ANA test results

- Clinicians need to be aware which ANA screening test (ANA IFA or solid-phase multi-analyte autoantigen arrays (SPMAA)) their laboratory uses.
- Does their laboratory report all intracellular staining (i.e., cytoplasmic and mitotic patterns) or only nuclear staining on the ANA IFA test?
- What is the sensitivity, specificity, commutability, and reference range of their ANA assay?
- In interpreting published reports on ANA, was the test performed on an inception or cross-sectional SLE cohort?
- In SLE, autoantibodies to more than one autoantigen are typically found, and the combinations of these various antibodies have important clinical associations.
- An important gap in ANA testing is the need for multicenter studies that use newer SPMAA on cohorts of very early or incomplete SLE.

serological evaluations are unlikely to provide a realistic picture of the B cell response in SLE.

Another factor impacting on ANA test results and interpretation is that the ANA IFA test lacks accuracy and commutability (traceability, harmonization) of results [23, 26]. Because achieving accuracy (closeness of a measured value to a standard or known value) of autoantibody assays is very challenging, assay precision (how close two or more assays are to each other or performance of an assay based on a predicate assay) is a common parameter used for reliable diagnostic assay design. Complete traceability [27, 28] and commutability [29] of ANA testing has yet to be achieved. As a caveat, although absolute standardization of autoantibody testing is generally recognized as extremely challenging, initiatives to improve standardization/harmonization within the wider field of in vitro diagnostics are underway [30].

One issue that contributes to the lack of harmonization and consensus in ANA IFA testing is whether *c*ytoplasmic and *m*itotic *p*atterns (CMP) should be considered as part of the ANA result. In an attempt to harmonize this definition of ANA, two separate international committees recommended that CMP should be included in the definition of ANA and that the nomenclature should be more appropriately changed to anti-cellular antibodies (ACA) [31, 32••]. In this manuscript, the term ANA (rather than ACA) is used simply because it is most widely recognized by clinicians. Nevertheless, it is important for the clinician to know if their diagnostic laboratory reports CMP staining in their ANA IFA results.

In addition, the harmonization of ANA IFA testing is challenging because other variables such as laboratory equipment (i.e., microscope optics and light sources), serum screening dilutions, and other factors have yet to be standardized [33, 34]. There is evidence that the recent introduction of automated microscopic testing provides more harmonized ANA IFA results [35–37]. However, this technology requires a quality assurance program that addresses the total ANA IFA process. For example, the ANA IFA is semi-quantitative at best and although immunofluorescence intensity units can be obtained, this approach has yet to be approved by regulatory agencies [23]. Next, although, in general, higher titer antibodies have better clinical correlations [38, 39], autoantibodies directed to different intracellular targets and differing IFA patterns have differing dilution curves, so that some ANA IFA systems (i.e., anti-U1RNP,

anti-Sm) may be regarded as high dilution/titer systems, whereas others (i.e., anti-SSA/Ro60, SS-B/La) are low dilution/titer systems [39]. Last, it should be appreciated that the ANA IFA test is only useful as a screening test and specific antibodies should be confirmed through the identification of specific antibodies using SPMAA [40].

Perhaps the significant challenges to harmonization, commutability, and traceability of the ANA IFA test are inconsequential because modern diagnostic laboratories are progressively migrating to SPMAA diagnostic platforms that have higher throughput and faster turn-around-times but, more importantly, utilize definable analytes/autoantigens in their test platforms [19, 41]. For the most part, SPMAAs are widely available and are currently used either as an approach to the diagnosis of specific SARDs (i.e., separate SLE, scleroderma (SSc), Sjögren's syndrome (SjS), autoimmune inflammatory myopathy (AIM) profiles), and/or the most common targets seen in SARDs included in a SARD screen as an alternative to the ANA IFA. However, there is some evidence that while SPMAA are a significant move forward, combining ANA IFA with SPMAA has higher clinical impact than either of the tests alone [3, 20, 36, 42, 43]. SLE serum samples that have negative SPMAA test results should ideally be tested by ANA IFA to determine if antibodies to targets not included in the SPMAA are detected.

Last, it is well known that SLE patients commonly have more than one autoantibody, typically reflecting what has been called "linked sets" [44] or B cell responses to macromolecular complexes such as spliceosomes, nucleosomes, and/or cytoplasmic ribonucleoprotein complexes. With the advent of SPMAA, it has been increasingly appreciated that combinations and permutations of autoantibodies in SLE can be associated with unique phenotypes. For example, a combination of anti-dsDNA and antinucleosome antibodies is reported to differentiate lupus nephritis (LN) from SLE without LN; anti-dsDNA plus anti-histone and antinucleosome antibodies are associated with a higher risk of severe LN than what can be attributed to the individual autoantibodies alone [45]. When anti-Ro52/TRIM21, anti-Ro60/SS-A, and anti-SS-B/La were found together, they were associated with xerostomia and xerophthalmia ($p<0.001$) [46]. Oral ulceration was associated with anti-Ro52/TRM21 and anti-Ro60/SS-A positivity but anti-SS-B/La negative (p 0.002) and alopecia was associated with anti-Ro60/SS-A positivity but anti-Ro52/TRIM21 and anti-SS-B/La negativity (p 0.003) [46]. Further, when anti-Ro52/TRIM21 antibodies were found in isolation, there was a negative association with xerophthalmia and photosensitivity with anti-Ro60/anti-La (p 0.003). These observations and others like them illustrate the limitations of interpreting the frequencies and clinical associations of any individual ANA result.

Specific autoantibodies

Since over 180 autoantibodies have been reported in SLE, a general context and consensus of only the key autoantibodies is discussed here. The frequency of the various autoantibodies is shown in Table 2. In the following discussion, when ANA IFA patterns on HEp-2 cells are referred to, they are cross-referenced to the corresponding AC-'X' nomenclature proposed by the International Consensus on Autoantibody Patterns (ICAP: https://anapatterns.org/index.php).

Table 2. Prevalence and clinical associations of SLE autoantibodies

Antibody target	Prevalence range* (%)	Prevalence in inception SLICC cohort (*n*=1049)** (%)	Clinical associations	Comments
dsDNA	30–70	40.5	CC, LN	Pathogenic: test results can vary depending on immunoassay used
Nucleosomes chromatin	20–70	N/A	LN, more severe disease progressive renal failure; disease activity; DIL	Pathogenetic: Note—many drugs originally associated with anti-histone and DIL are no longer in wide use; harder to obtain validated assays because of difficulty standardizing the target macromolecular nucleocomplex
Histone	10–80	40.1	SLE, drug-induced SLE; NPSLE	Frequency depends on assay and which histones are included; higher titers, especially anti-H2B, anti-H4, acetylated H4, and H2A more specific for SLE
High-mobility group proteins	20–49	N/A	SLE disease activity	Sensitivity and specificity varies depending on the HMG protein being studied.
Dense fine speckled 70	1–10	7.1	Monospecific antibodies rare in SLE	Reported in variety of autoinflammatory syndromes and health individuals. Monospecific antibodies may be used to ruling out diagnosis of SLE and other SARD.
Sm (U2-U6 RNP)	5–30	24.7	CC, serositis, LN, NPSLE, CSF, NPSLE	Predictive: SmD3 containing a symmetrical dimethylarginine at position 112 most specific for SLE
U1-RNP	15–50	32.4	Leukopenia; NPSLE; ILD: IgM anti-U1RNP antibodies predominant in SLE compared to IgG anti-U1RNP without IgM more frequent in MCTD	Anti-U1RNP in CSF 64.3% sensitivity and 92.9% specificity for NPSLE
Ribosomal P	10–30	16.1	Renal disease; malar rash; possibly NPSLE; antibodies to C22 peptide highly specific for SLE	Associated with anti-dsDNA; although C22 of protein has the most specific SLE

Table 2. (Continued)

Antibody target	Prevalence range* (%)	Prevalence in inception SLICC cohort (*n*=1049)** (%)	Clinical associations	Comments
				epitope, not all assays utilize this peptide in their assays; prevalence varies in different countries
SS-A/Ro60	25–60	47.3	SCLE, C4 deficiency, NLE; in pediatric SLE milder disease (cutaneous, MSK)	Predictive: titers important in NLE
Ro52/TRIM21	10–40	35.9	Leukopenia; ~70% of patients with anti-Ro52 but not anti-Ro60-antibodies have SARD (majority have UCTD followed by SLE); ANA negative SLE	Seen in patients with malignancy with or without evidence of SARD
SS-B/La	5–25	15.9	SjS, SCLE; NLE, leukopenia, serositis; rare in pediatric SLE	Protective: less renal disease
C1q	15–60	N/A	Lupus nephritis; prevalence 13% in controls	Simultaneously positive anti-C1q, anti-dsDNA and low complement was strongly associated with renal involvement
Ku	5–20	N/A	Not specific for SLE; SSc and myositis overlap; other SARD; Raynaud's, myositis, arthritis; UCTD	Ku is a heterodimer consisting of 70 kDa (p70) and ~80 kDa (p80) protein subunits that binds blunt-ends of x-ray damaged DNA.
PCNA	0.5–5	7.3	More severe SLE; may be transiently expressed and/or decreased with therapy	PCNA is the auxiliary protein of DNA polymerase delta component of a macromolecular complex when IFA and SPMAA, specific for SLE.
Cardiolipin	20–60	12.6	CC, APS; thrombosis; pulmonary hypertension	Predictive: decreased survival
AMA M5	~25	N/A	APS	Closes serological gap in APS; thrombocytopenia, fetal loss, lupus anticoagulant; associated with anticardiolipin, anti-β2GP1, and biologically false-positive VDRL
β2GP1	30–45	14.0	CC, APS	Predictive of thrombosis and fetal loss: closes

Table 2. (Continued)

Antibody target	Prevalence range* (%)	Prevalence in inception SLICC cohort (*n*=1049)** (%)	Clinical associations	Comments
				serological gap in APS; glycosylation of Fc domain of anti-β2GP1 may be important in pathogenesis
β2GP1 domain 1	40–56	N/A	APS	Higher specificity than full length β2GP1; IgA antibodies important
PS/PT	30–54	N/A	APS; correlated with lupus anticoagulant and increased risk of thrombosis	May be a surrogate biomarker for lupus anticoagulant. Commercial kit available.

AMA M5 anti-mitochondrial M5 type antibodies, *APS* antiphospholipid syndrome, *β2GP1* beta 2 glycoprotein 1, *CC* classification criteria, *CI* confidence interval, *CSF* cerebrospinal fluid, *DIL* drug-induced lupus, *dsDNA* double-stranded DNA, *HMG* high mobility group, *ILD* interstitial lung disease, *kDa* kilodalton, *LN* lupus nephritis, *SPMAA* multi-analyte antigen array, *MCTD* mixed connective tissue disease, *MSK* musculoskeletal, *N/A* not assessed, *NLE* neonatal lupus erythematosus, *NPSLE* neuropsychiatric SLE, *OR* odds ratio, *PCNA* proliferating cell nuclear antigen, *PS/PT* phosphatidyl serine/prothrombin complex, *RNP* ribonucleoprotein, *SARD* systemic autoimmune rheumatic disease, *SCLE* subacute cutaneous lupus erythematosus, *SjS* Sjögren's syndrome, *SS-A*, *SS-B* Sjögren syndrome antigens A and B, *SSc* systemic sclerosis, *TRIM* tripartite motif, *UCTD* undifferentiated connective tissue disease, *VDRL* Venereal Disease Research Laboratory
*For interpretation, see Table 1 and section "Important considerations in interpretation of ANA test results and reports." **Reference [8•]

Chromatin components

Autoantibodies directed against chromatin components are typically associated with a homogenous (AC-1) or dense fine speckled (DFS) pattern (AC-2) on HEp-2 IFA. Exceptions are antibodies directed specifically to histone (H1), the inner core histones (H3, H4), and high-mobility group (HMG) proteins , which have no known consistent IFA pattern.

Double-stranded DNA

Antibodies directed to double-stranded DNA (dsDNA) were one of the first, disease-specific B cell targets described in SLE (reviewed in [47••, 48]). Anti-dsDNA antibodies are typically associated with LN [49] and can be used to monitor disease activity [47, 50]. It is important to appreciate that the anti-dsDNA system is highly complex and this, in part, is reflected by the performance of different immunoassays and diagnostic platforms used to detect them. Historically, the Farr radioimmunoassay was preferred because it reputedly measured primarily high affinity antibodies and hence reflected a more "trained" B cell response in addition to antibodies that likely had pathogenic potential in LN. The Farr assay has become decreasingly used because of safety concerns with assays that employ radioisotopes and there was a tendency of the kits to be in short supply. Hence, many laboratories reverted to other assays such as addressable laser bead immunoassay (ALBIA), enzyme-linked immunosorbent assay (ELISA), chemiluminescence immunoassay (CIA), and the *Crithidia luciliae* IFA

[47]. One ongoing issue is whether a negative ANA IFA result is a "false negative" when the results of any of the SPMAA anti-dsDNA tests listed above are positive. To address this apparent paradox, it is important to recognize the highly complex nature of dsDNA and appreciate that in cells (i.e., HEp-2) where dsDNA is bound by histones, HMG proteins, and other DNA-binding proteins and stabilized with organic fixatives, there is no a priori reason to expect complete agreement of results ANA IFA and SPMAAs when the latter use purified or synthetic dsDNA. For a more thorough discussion of limitations of anti-dsDNA testing, the reader is referred to a recent review [47••].

Histones

Histones are a class of low molecular weight, cationic proteins that are bound to nuclear dsDNA. Histones can be subclassified as cross-linkers of DNA and the nucleosome (H1) or components of the core nucleosome body (H2a, H2b, H3, and H4) [51]. Methylated, acetylated and other posttranscriptional modified histones are now known to be critical in the epigenetic gene expression [52]. In addition, they are components of apoptotic bodies [52, 53] and neutrophil extracellular traps (NETS) [54], which are considered to be important pathogenic phenomena in SLE [55]. As compared to antibodies to the core histones, antibodies directed to H1 and its variants are the least specific for SLE. Although antibodies to histones and nucleosome have been primarily linked to drug-induced lupus (DIL) [55, 56], it is important to appreciate that this association was more relevant to drugs that are no longer in wide use (e.g., hydralazine, procainamide) compared to the biological therapeutic-induced DIL that is more prevalent today. Since histones are key components of the nucleosome, antibodies to the nucleosome are considered more diagnostically relevant.

Nucleosomes

The terms antinucleosome and anti-chromatin antibodies tend to be used interchangeably; however, an important distinction is that although the term "chromatin" encompasses nucleosomes, nucleosomes are more restricted in their molecular structure(s) and components, the latter largely relegated to dsDNA complexed to histones as described above, while chromatin contains dsDNA, HMG proteins, and other gene-regulatory elements [51]. Hence, most commercially available assays are based on purified nucleosomes that have been further processed to remove the inter-nucleosome linker DNA as well as less avidly bound proteins such as HMGs and H1. In this format, antinucleosome antibodies are highly, but not exclusively, correlated with anti-dsDNA and are highly specific for SLE [51, 57]. In some studies, nucleosomes and antinucleosome antibodies are thought to be more pathogenic than anti-dsDNA. Antinucleosome antibodies have been reported to antedate the appearance of anti-dsDNA and predict the development of SLE in patients who developed renal failure [58] and antiphospholipid syndrome (APS) [59]. Despite numerous publications indicating the importance of antinucleosome antibodies as biomarkers for SLE [51, 60, 61], other less favorable views have been offered [62•].

High-mobility group proteins

HMG proteins are a family of low molecular weight, chromatin-associated proteins that, except for HMG box protein-1 (HMGB1) and HMG box protein-2 (HMGB2), primarily bind to linker (i.e., non-nucleosome) DNA. Although HMGB1 is typically associated with nucleosomes, it also shuttles between the nucleus and the cytoplasm. HMGB1 consists of three separate domains: Box A, Box B, and an acidic tail of which Box A acts as a competitive antagonist for HMGB1 and has been considered as a potential treatment option for SLE [63]. Much interest has focused on HMGB1 because it is secreted by inflammatory cells and passively released from apoptotic and necrotic cells, where it has pro-inflammatory effects [64, 65]. It has been suggested that HMGB1 helps elicit anti-dsDNA antibody production in SLE [66]. The titers of anti-HMGB1 antibodies correlated with anti-dsDNA antibody levels (r=0.49; p<0.001) and less pronounced correlations were observed with SLE disease activity index (SLEDAI-2K) (r=0.15; p=0.04), low C4 (r=–0.23; p=0.002), low C3 levels, and proteinuria [63, 67]. These observations suggested that HMGB1-anti-HMGB1 immune complexes play a role in the pathogenesis of LN. Titers of anti-Box A antibodies were also increased in 73% of patients with LN and 71% of non-LN flares. It was concluded that antibodies to the HMGB1 Box A domain of might be a novel biomarker for SLE.

Anti-HMGB1/HMGB2 antibodies were detected in SLE, rheumatoid arthritis (RA), SjS, and SSc [66]. Of interest, HMGBI and HMGB2 may be target antigens of perinuclear neutrophil cytoplasmic antibodies (pANCA). However, anti-HMGB1 antibodies were not associated with ANCA-associated vasculopathies [68]. Autoantibodies to another HMG protein, such as the structure specific recognition protein I (SSRP1) was identified by ELISA and western blot in 28% of SLE sera, compared to 8.3% of healthy individuals but not in other SARD [69].

Dense fine speckled

The typical DFS IFA staining pattern is recognized as uniformly distributed fine speckles throughout interphase nuclei and on metaphase chromatin (AC-2) (reviewed in [70••, 71]). The target antigen associated with the DFS IFA pattern was first identified as dense fine speckled 70 (DFS70) based on the apparent molecular weight of 70 kDa, but the protein was later recognized as the lens epithelium–derived growth factor (LEDGF) and more recently as the DNA-binding transcription coactivator p75 [72]. Since the first report in interstitial cystitis, anti-DFS70 antibodies have been reported in patients with a variety of chronic inflammatory conditions, cancer, and most notably in healthy individuals [70••, 71, 73]. Anti-DFS70 antibodies are now recognized as one of the key targets in high titer positive ANA IFA healthy and non-SARD sera [74]. Isolated (monospecific or no other detectable autoantibodies) anti-DFS70 antibodies described in healthy individuals were rarely found in SLE but were found associated with SLE-related autoantibodies in a small proportion of sera [75]. Hence, the presence of anti-DFS70 in SLE sera is currently thought to have limited clinical value. A recent report indicates geographic differences in the prevalence of anti-DFS70 antibodies and that they occur preferentially in females and young individuals [76].

Small nuclear ribonucleoproteins: Sm and U1-RNP

Autoantibodies directed against small nuclear ribonucleoproteins (snRNP) components (Sm, U1-RNP) are typically associated with nuclear speckled (AC-4; AC-5) patterns on HEp-2 IFA. Anti-Sm antibodies are highly specific for SLE. By comparison, although anti-Sm and anti-U1RNP commonly coexist in SLE, anti-U1RNP antibodies are found in patients with a variety of other SARD (reviewed in [77]). Although anti-Sm is one of the most widely requested autoantibody tests, it lacks sensitivity because it is present in only 5–30% of SLE patients [78]. Nevertheless, largely because of its high specificity, it is one of the serologic criteria in the ACR [79] and SLICC [80] Classification Criteria for SLE. Although anti-Sm is associated with classical SLE, it is also seen in patients with SLE-overlap syndromes [77]. Anti-Sm antibodies have been described in patients without SLE, although follow-up studies showed that some eventually developed that disease [77]. These observations highlight the importance of Sm and other SLE-specific autoantibodies as predictors of SLE [81]. The titers of anti-Sm antibodies have been reported to correlate with disease activity, milder renal and central nervous involvement, or late-onset LN, but these associations are controversial [7]. Twenty-five to forty percent of anti-U1RNP-positive SLE patients also have anti-Sm, although this varied in different cohorts [78]. Even though anti-U1RNP antibodies are found in a high proportion of SLE, they are also reported in SSc, AIM, SjS, and other SARD. By comparison, all (~100%) patients with mixed connective tissue disease (MCTD) are, by definition, positive for anti-U1RNP antibodies. However, anti-U1RNP antibodies are not a specific biomarker for MCTD. Regardless of the disease association, anti-U1RNP-positive patients typically have Raynaud's phenomenon, swollen digits, and leukopenia [82].

DNA-dependent protein kinase (DNA-PK/Ku)

Ku is a heterodimer composed of 70 and ~85 kDa non-histone nuclear proteins. This antigen has gained research interest because it is a DNA-dependent protein kinase (DNA-PK) that is critical in the repair of double-strand DNA breaks induced by ionizing radiation [83]. Antibodies to various components of this macromolecular DNA-PK complex have been reported in SLE and related SARD [84]. Although initially thought to be specific for polymyositis/SSc overlap syndrome [85], subsequent studies demonstrated that anti-Ku antibodies are also found in SLE, MCTD and SSc [86–88]. Additional studies revealed that anti-Ku antibodies were also detected in a variety of other SARD and were associated with polymyositis/SSc overlap syndrome in Japanese cohorts but with SLE and overlap syndromes in African-American cohorts [87]. In a small Japanese polymyositis/SSc overlap syndrome cohort (n=11), precipitating autoantibodies to Ku were found in 55% as compared to 6% of Japanese SLE patients and <1% of other SARD [85]. In African-American individuals, these antibodies were most strongly associated with SLE (14%), whereas in Caucasian people, anti-Ku antibodies are rare regardless of the clinical diagnosis [87]. Overall, anti-Ku autoantibodies are relatively rare, being reported in 4% of patients with MCTD/overlap syndrome and in <1% of SLE, and even rarer in other SARD. More multicenter studies of inception cohorts of SLE and other SARDS using newer diagnostic platforms that include Ku as an analyte are needed.

Other nuclear targets

Sjögren syndrome antigen B/La

Autoantibodies directed against Sjögren syndrome antigen B (SS-B)/La are highly specific (~90%) and sensitive (~85%) for SjS [89, 90]. The prevalence of these antibodies is also high in mothers that give birth to infants with neonatal lupus syndrome (NLS) [91]. In NLS, approximately 10–15% of cases of congenital heart block are not exposed to anti-Ro60/SS-A or SS-B/La [91] while the cutaneous manifestations of NLS were reported in infants exposed only to anti-U1RNP antibodies [91]. It was reported that anti-SS-B/La and Ro60/SS-A antibodies can be detected 2.8 years before the onset of the symptoms and 3.6 years before diagnosis of SLE [92], suggesting a predictive role. SLE patients that have anti-SS-B/La antibodies usually develop secondary SjS syndrome. Anti-SS-B/La autoantibodies are rarely found alone, usually coinciding with anti-Ro60/SS-A autoantibodies [90].

Cytoplasmic

Ribosomal P

Autoantibodies to ribosomes were first described in the 1960s in SLE sera followed by identification of key ribosomal proteins (Rib-P) in the 1970s [93, 94] and three of the ribosomal phosphoproteins (P0, P1, P2) in the 1980s [95, 96]. The Rib-P are largely localized to the cytoplasm and are components of the 60S ribosomal subunit [93]. Over the years, anti-Rib-P antibodies have been the subject of extensive study and have been shown to be a highly specific biomarker for the diagnosis of SLE [97–99]. They are associated with LN, autoimmune hepatitis and, although controversial, with neuropsychiatric SLE (NPSLE) subset [100, 101]. Experimental evidence indicates that anti-Rib-P have a pathogenic role in LN and NPSLE. Despite remarkable evidence for their high disease specificity, anti-Rib-P have not been included in classification or diagnostic criteria for SLE. A significant challenge in interpreting the published literature is the variability of diagnostic platforms used to detect anti-Rib-P and the antigens included in the assays. Evidence indicates that the major epitope is localized to the C-terminal 22 amino acids [98, 102], and some diagnostic assays use this analyte in their assays but other assays use various combinations of the other Rib-P's [103, 104]. This likely accounts for the marked discrepancies in frequencies (10–47%) and association with clinical and demographic features reported in SLE cohorts. Although Rib-P proteins are localized in the cytoplasm, ANA IFA is not a reliable screening method to detect them [104, 105], but when it is positive, it is typically associated with a cytoplasmic AC-19 pattern. Anti-Rib-P antibodies commonly coexist with anti-dsDNA and anticardiolipin (aCL) antibodies [106, 107] and this may account with the observed association with LN and NPSLE.

Ro60/SSA

In general, anti-Ro60/SS-A antibodies are not reliably detected by most ANA IFA screening assays. An exception is a specialized HEp-2 substrate (HEp-2000, Immuno Concepts) that has been transfected with the Ro60/SS-A gene where it is overexpressed in some cells and more reliably detected by IFA [108]. Depending

upon the method of detection and the cohort being studied, anti-Ro60/SS-A is found in up to 50% of patients with SLE. By comparison, the prevalence of anti-Ro60/SS-A is up to 85% in SjS. The prevalence of anti-Ro60/SS-A is similarly high in mothers of infants with NLS and subacute cutaneous lupus. A much lower prevalence (<25%) of anti-Ro60/SS-A is seen in SSc, AIM, RA, and autoimmune liver diseases. In SLE, anti-Ro60/SS-A is associated with photosensitive skin rash, elevated serum immunoglobulins, lower complement levels, lymphopenia, and leukopenia. In SLE the combination of anti-Ro60/SS-A and anti-SS-B/La may be protective against neurologic and kidney disease among patients [109]. Antibodies to anti-Ro60/SS-A have been reported to be one of the earliest antibodies to appear years before a diagnosis of SLE is made [92].

Ro52/TRIM21

Ro52/TRIM21 is a 52-kDa member of the tripartite motif-containing (TRIM) super family of proteins, is a factor in innate and acquired immunity and pathological autoimmune processes, and is an interferon-(IFN-) inducible protein in some cells [110]. Its role in pathological autoimmune processes includes the aberrant ubiquitylation of the interferon-regulatory factor (IRF) family of proteins, regulation of type I interferon and pro-inflammatory cytokines [110, 111], is a key effector in the toll-like receptor 3 pathway [112], and is linked to IL-23 and BAFF expression [111]. Hence, Ro52/TRIM21 has been purported as an attractive therapeutic target for SLE [110]. Anti-Ro52/TRIM21 antibodies are not detected by conventional HEp-2 ANA IFA.

Ro52/TRIM21 was initially reported to be part of the same antigenic macromolecule as Ro60/SS-A and the B cell response to these two targets were strongly linked (reviewed in [113]) Subsequently, both of these claims were shown to be incorrect, but the medical literature is confounded because many commercially available diagnostics assays included both Ro52/TRIM21 and Ro60/SS-A in a single test and many reports of clinical associations linked both targets together. As a result, detailed studies using anti-Ro52/TRM21 as a separate analyte are now needed. When this has been done, anti-Ro52/TIRM21 was found to be not specific for any SARD [114], but in SSc and MCTD, were reported to be associated with polyautoimmunity (i.e., patients that have more than one autoimmune condition), interstitial lung disease, rheumatoid factor [114, 115], and in SjS with more aggressive disease [116]. In SLE using line immunoassay (LIA) and ELISA, monospecific anti-Ro52/TRIM21 was observed in 6/67 (5.9%) and was most commonly associated with anti-Ro60/SS-A (22/67, 32.8%) [114]. In other studies, this autoantibody was associated with leukopenia and ~70% of patients with anti-Ro52/TRIM21 but no anti-Ro60/SS-A antibodies had undifferentiated connective tissue disease (UCTD) or SLE [46, 117, 118].

Cell cycle

Proliferating cell nuclear antigen

Antibodies directed to the 35-kDa proliferating cell nuclear antigen (PCNA) were first described in a Japanese female with SLE (reviewed in [119]). The identification of anti-PCNA and its purported high specificity

for SLE was based on a unique cell cycle IFA pattern on HEp-2 cells (AC-13) and a specific immunoprecipitation line in double immunodiffusion. However, subsequent studies that used other assays such as LIA, ELISA, and ALBIA [119] indicated that anti-PCNA antibodies were not specific for SLE [120]. Further studies (unpublished) indicate that the high specificity of anti-PCNA can be retained if both the typical IFA AC-13 pattern and high titer anti-PCNA antibodies are detected by SPMAA [121]. The disadvantage of anti-PCNA is that it has low sensitivity (<5%) for SLE and it may disappear after immunosuppression treatment [119].

Extracellular

C1q

Anti-C1q antibodies are predictive of and associated with LN, especially membranoproliferative disease [122•, 123, 124]. In SLE with active LN, anti-C1q antibodies were found in up to 90% of patients [124, 125]. The combined detection of C1q and dsDNA antibodies predicted flares of LN and have a combined sensitivity of 91% and specificity of 90% for LN [126]. Anti-C1q antibodies are as specific as high avidity dsDNA antibodies for LN and close a diagnostic and serological gap in some cases [122, 127].

Phospholipids and related antigens [128]

Autoantibodies directed to phospholipids (aCL, beta2 glycoprotein I (β2-GPI) and phospholipid-related targets (phosphatidyl serine/prothrombin complex or PS/PT) are a heterogeneous family of autoantibodies that are an important serological criteria in the classification of SLE [79, 80] and APS [128, 129]. Historically, anti-phospholipid antibodies (aPL) antibodies are traced to the identification of a biologically false-positive test for syphilis and the eventual link to cardiolipin, other anionic (negatively charged) phospholipids, and phospholipid binding proteins [2, 128]. Some studies indicate that they also have predictive value [130].

Cardiolipin

The importance of aCL antibodies in SLE and APS has had a rather tumultuous history. However, aCL remains a key classification criterion and has a prominent place in the laboratory diagnosis of both diseases. The primary use of the aCL assay is as an adjunct to anti-β2-GPI and lupus anticoagulant (LA) tests [128]. Its importance was underscored by a study reporting that >25% of APS patients were negative for anti-β2-GPI and LA but positive for aCL [131]. Hence, it remains an important assay to close the serological gap and classification of APS. IgG and IgM aCL test results should be reported as IgG anti-phospholipid units/mL (GPL)" and IgM anti-phospholipid units/mL (GPL)" units derived from calibrators composed of a pool of polyclonal aPLs. A positive aCL result is defined as a medium or high titer of greater than 40 GPL or MPL units. As an alternative, assay cutoffs established at greater than the 99th percentile of results obtained from a control group of at least 50 individuals is considered acceptable [79]. A significant limitation of the aCL test is its lack of specificity as it can be positive in other diseases, particularly those of infectious origin.

β2-glycoprotein 1

β2-GPI consists of 326 amino acids and approximately 20% of the molecule is comprised of carbohydrates. β2-GPI antibodies are reported to have a higher specificity but lower sensitivity for APS than aCL antibodies. High titer IgG- and IgM-isotype are diagnostic biomarkers and classification criteria of APS [128, 129] where they are associated with vascular thrombosis and recurrent spontaneous abortion. The value of anti-β2-GPI antibodies has highest clinical value when they are detected on two or more occasions at least 12 weeks apart. The prevalence and levels of anti-β2-GPI antibodies are significantly higher in SLE with arterial or venous thrombosis than in SLE patients without these complications and represent a significant risk factor for arterial thrombosis in SLE [132]. IgA anti-β2-GPI antibodies are reported to be more prevalent than IgG or IgM isotypes. The presence of both β2-GPI and aCL antibodies is strongly associated with clinical symptoms of APS [133, 134]. Unlike aCL antibodies, anti-β2-GPI antibodies are seldom detectable in infectious diseases. β2-GPI is composed of five molecular domains, of which the exposed residues spanning the region from arginine (Arg)39 to Arg43 of domain 1 (DI) represent the primary epitope bound by APS autoantibodies. The pathogenic subsets of anti-β2-GPI associated with thrombosis are reported to also bind DI. An approach to increasing the specificity of anti-β2-GPI testing was the development of a commercial immunoassay that included only DI of β2-GPI [134–136]. Recent reports indicate the value of this assay in risk management [135] and predicting thrombosis and late pregnancy morbidity [137]. However, as a word of caution, there is variation of results in different commercially available assays [138].

Non-criteria autoantibodies: phosphatidylserine/prothrombin complex

Despite over 40 years of study and research, numerous difficulties are attributed to the APS criteria assays. In particular, no single or combination of APS criteria antibodies are present in up to 25% of patients who are strongly suspected of having APS, resulting in a "seronegative gap" [128, 139]. A number of non-criteria autoantibodies, such as antiphosphatidylethanolamine, antibodies to proteins of the coagulation cascade, and antibodies to the PS/PT complex, have been associated with APS [128, 140, 141]. Some interest has focused on the mitochondrial membrane antigen M5 which is associated with thrombocytopenia and fetal loss, and coexists with LA, aCL, anti-β2GP1, and biologically false-positive VDRL [142, 143]. With the availability of a commercial anti-PS/PT assay [144], a number of reports have focused on its value [145], particularly its use in closing the seronegative gap in APS [141, 146]. Although the presence of anti-PS/PT antibodies appear to be independent of the LA, they may be a potential substitute for the troubled LA assay [146, 147]. The clinical utility of these assays, however, is limited by a paucity of prospective clinical data and lack of standardization [138]. Therefore, additional studies are required before any "noncriteria" immunoassays are included in standard APS classification criteria.

Orphan autoantibodies

As noted above, more than 180 autoantibodies have been described in SLE and the list continues to grow. These "orphan" autoantibodies [81, 130] were not discussed here but the reader is referred to published studies of selected

"orphan" autoantibodies [7, 148]. The reason that many of these antibodies are not commonly used is that they have yet to meet SMAARTT (specificity–sensitivity, measurable, actionable, added value, realistic, titres, timely) criteria for development by laboratory diagnostics or met with demand from clinical practitioners [149•]. As SPMAAs become increasingly available, it is possible that some of these "orphan" biomarkers will find new uses in the prediction, diagnosis, and prognosis of SLE.

Summary

In summary, there is a need for evidence-based and unbiased approaches to ANA testing. In parallel, a compelling case can be made for attenuation of significant morbidity and health care expenditures in SLE by using ANA testing for "case finding" and making a much earlier diagnosis and prevention of high morbidity [150]. Given the shortcomings of ANA IFA testing [151], it appears that there should be a move to SPMAAs as the screening tests of choice for SLE.

Compliance with Ethical Standards

Conflict of Interest
Dr. Fritzler reports personal fees, non-financial support, and others from Inova Diagnostics Inc., others from Alexion Canada, personal fees from Werfen International, outside the submitted work. Dr. Choi declares that she has no conflict of interest.

Human and Animal Rights and Informed Consent
This article does not contain any studies with human or animal subjects performed by any of the authors.

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