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Perspective: Widening Spectrum and Gaps in Autoantibody Testing for Systemic Autoimmune Diseases

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ABSTRACT

Over half a century has elapsed since the introduction of ANA and autoantibody testing to confirm the diagnosis of and screen for systemic autoimmune diseases (SAID). Despite this long history, there are several gaps in the understanding, utilization and interpretation of the test results. One reason for persisting gaps is the proliferation of novel autoantibody targets described along with a widening spectrum of SAID and clinicians that use these tests. The gaps include standardization of ANA test protocols and the availability of appropriate controls. There are also persisting nomenclature gaps. A significant proportion of SAIDs are seronegative, a gap that is being closed by ongoing research and discovery.

Keywords: Autoantibodies, Anti-nuclear antibodies, Diagnostic assays, Systemic autoimmune diseases

Abbreviations: ALBIA: Addressable Laser Bead Immunoassay; ALD: Autoimmune Liver Diseases; AE: Autoimmune Encephalitis; ANA: Anti-Nuclear Antibody; ANCA: Anti-Neutrophil Cytoplasmic Antigen; APS: Anti-Phospholipid Syndrome; AQP4: Aquaporin 4; IIF: Indirect Immunofluorescence; CENP: Centromere Protein; DID: Double Immunodiffusion; DFS: Dense Fine Speckled; DNA: Deoxyribonucleic Acid; ELISA: Enzyme Linked Immunoassay; GN: Glomerulonephritis; GP1: Glycoprotein 1; IIM: Idiopathic Inflammatory Myopathies; ILD: Interstitial Lung Disease; Jo-1: Histidyl tRNA Synthetase; LIA: Line Immunoassay: LE: Lupus Erythematosus; MAA: Multi-Analyte Array; MAAAA: Multi-Analyte Array with Algorithmic Analysis; MCTD: Mixed Connective Tissue Disease; NLE: Neonatal Lupus Erythematosus; NMDAR1: N-Methyl-D-Aspartate Receptor 1; NPLE: Neuropsychiatric Lupus Erythematosus; OS: Overlap Syndrome; PCNA: Proliferating Cell Nuclear Antigen; PLA2R: Phospholipase A2 Receptor; PM/Scl: Polymyositis/Scleroderma Antigen; Rib-P: Ribosomal P Protein; RNAP: RNA Polymerase; RNP: Ribonucleoprotein; SACLE: Subacute Cutaneous Lupus; SAID: Systemic Autoimmune Diseases; Sm: Smith Autoantigen; SjS: Sjögren's Syndrome; SLE: Systemic Lupus Erythematosus; SNP: Soluble Nucleoprotein; SPA: Solid Phase Assays; SSA: Sjögren's Syndrome Antigen A; SSc: Systemic Sclerosis; Th/To: Mitochondrial RNA Processing Complex, Topoisomerase I (Scl-70); TRIM: Tri-Partite Motif; UCTD: Undifferentiated Connective Tissue Disease

INTRODUCTION

I recently attended a medical meeting focused on a systemic autoimmune disease (SAID) and as the meeting considered metanalyses, classification criteria, odds ratios, confidence intervals, dimethyl 'chicken-wire' and 'kappaphredon' levels, it became clear that despite more than a half century of study and experience, gaps in autoantibody testing, especially the anti-nuclear antibody (ANA) test and its clinical value, are not largely resolved **(Table 1)**. Some of these gaps persist despite knowledge available to fill them, while other gaps require additional effort and international collaboration. The perspectives in this article focus on the drivers of the gaps, the gaps themselves and where possible, resolutions to the gaps.

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Table 1. Gaps in autoantibody testing.

GAP	Comment	Resolution
Standardization	Lack of standardized screening dilutions, secondary antibodies, microscopes and manufacturing protocols	May be easier to standardize MAA in the future.
Assay Performance	Gap between ANA HEp-2 IIF and high-throughput SPA, lead to the former being declared the 'gold standard' ANA screening test	Industry has improved the performance of SPAs so that some are equivalent to or exceed performance characteristics of ANA HEp-2 IIF [14-16]. Development of automated, robotic and digital ANA IIF technologies has improved performance [17].
Nomenclature	Autoantibody systems such as SSA/Ro60 and Ro52/TRIM21 are often confused and/or regarded as one; IIF pattern nomenclature	Journals and reviewers need to require a distinction between SSA/Ro60 and Ro52/TRIM21. International Consensus on Autoantibody Patterns (ICAP) a significant step forward [12,27].
Seronegative SAID*	Up to 30% of some SAID are 'seronegative'	Research continues to identify SAID-related and SAID-specific autoantibodies. Gap will be closed when key targets are included in MAA to achieve a sensitivity of >90%.
Disease Classification Criteria	Historic disease classification criteria have been silent with respect to performance characteristics of tests used to identify autoantibodies included in criteria	SLICC criteria specified anti-dsDNA assay cutoff at 2X upper limit of normal [53]. In newer EULAR/ACR Criteria [51], ANA is a required entry criterion and is specified to have sensitivity of >90%.

Abbreviations: ANA: Anti-Nuclear Antibody; IIF: Indirect Immunofluorescence; MAA: Multi-Analyte Arrays; SAID: Systemic Autoimmune Diseases; SPA: Solid Phase Assays; SSA: Sjögren's Syndrome Antigen A; TRIM: Tri-Partite Motif

Widening spectrum: 'Clientele', autoantibody: Discoveries and technologies

Arguably, the major change in autoantibody testing over the past half century has not been the continuous discovery of novel autoantibodies as biomarkers for various SAID or steady advances in diagnostic technologies, but the broadening spectrum of clinicians and health care providers that use and rely on autoantibody testing in their practices **(Figure 1)** [1]. Dating to the discovery of the LE cell and the development of the LE cell test [2] and then the indirect immunofluorescence (IIF) assay using cryopreserved rodent tissue sections [3], the initial autoantibody testing 'clientele' was largely restricted to rheumatologists and immunologists who were attending to patients with various stages of systemic lupus erythematous (SLE). The onset of the era of cell and molecular biology in the mid-1960s became an inflection point for a widening spectrum of autoantibodies associated with SAIDs, such as anti-Sm for SLE [4] and anti-U1-ribonucleoprotein (RNP) for mixed connective tissue disease (MCTD) [5]. By the mid-1970s, the next

major inflection came when the advantages of tissue culture cells, such as HEp-2, became obvious and they were very rapidly adopted by the diagnostic industry as an alternative to rodent cryopreserved sections for the ANA test [6]. Prior to that, IIF on rodent tissue sections was considered an "insensitive" screening test for systemic sclerosis (SSc), Sjögren's syndrome (SjS), autoimmune inflammatory myopathies (AIM) and other SARDs because, other than nuclear speckled and nucleolar patterns observed in IIF tests of SSc sera, more common autoantibodies, such as anti-centromere, anti-RNA polymerase, anti-Ro60/SSA and -La/SSB that came to light with the introduction of HEp-2 substrates, were typically not seen **(Table 2)**. These inflection points marked the beginning of the 'golden age of autoantibody discovery' and along with that, the spectrum of clinicians that utilize the ANA and related autoantibody tests has expanded to include primary care providers and virtually every subspecialty in medicine including, most recently, pulmonary medicine and psychiatry **(Figure 1)**. Hence, SAIDs and their associated biomarkers have gained prominence in virtually all branches of medicine, a spectrum that will likely continue to widen. In addition, with this widening spectrum of autoantibodies and clinician 'clientele', the ANA and other autoantibody tests became known as screening tests rather than 'confirmatory diagnostic tests' with the result being decreasing pre-test probability **(Figure 1)** and concerns about inappropriate use of ANA testing [7].

The broadening spectrum of SAID is intertwined with newer high-throughput solid phase assays (SPA) such as enzyme linked immunoassays (ELISA), addressable laser bead immunoassays (ALBIA) and line immunoassays (LIA) developed as substitutes for the ANA IIF [8]. Because of perceived unsatisfactory performance characteristics (i.e., lack of sensitivity) of some SPAs, the ANA IIF test was declared the 'gold standard' screening test in 2010 [9,10]. In that declaration it was not clear that the wide spectrum of SAIDs was taken into consideration. For example, it is well-known that the IIF HEp2 test has limited (i.e., <60%) sensitivity for SjS, AIM, anti-phospholipid syndrome (APS) and the broader spectrum of SAIDs **(Table 2)**. Notable exceptions where the HEp-2 assay has higher (>80%) sensitivity or other clinical utility include SLE, SSc, autoimmune liver diseases and uveitis associated with juvenile idiopathic arthritis [11,12].

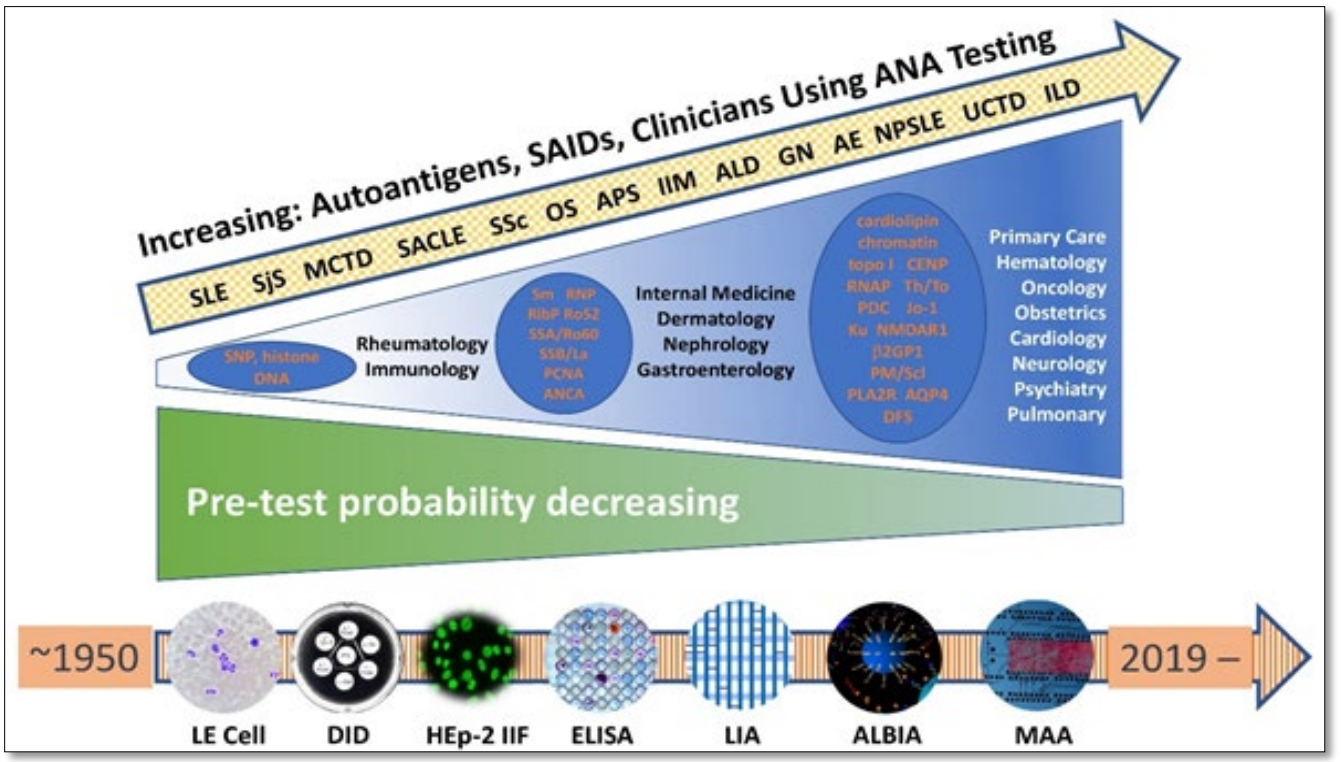

Figure 1. The progression of ANA and autoantibody testing in SAID dating from the discovery of the LE cell to the present, is characterized by a proliferation of autoantibody targets, increased widening of the spectrum of clinicians that use the test but a decline in the pre-test probability of patients being tested. ANA IIF and now ELISA, ALBIA and MAA as screening tests are routinely used for SAIDs with an emerging focus of very early SAID.

Table 2. Spectrum of autoantibodies and serological gaps in systemic autoimmune diseases (SAID).

SAID	Current Key Autoantibodies Available in Commercial Kits	Serological Gap (%)	Comment	Review References
SLE	dsDNA Sm (U2-U6 RNP) U1 RNP Chromatin/nucleosomes Histone Ribosomal P protein SS-A/Ro60 C1q PCNA Ro52/TRIM21 Ku	5-10%	Variation in gap dependent on variables in SLE cohorts studied: • Classification criteria • Diagnostic criteria • Cross-sectional vs. Inception	[21,35,54]
SSc	Centromere proteins (A/B) Topoisomerase 1 (Scl-70) RNA polymerase PM/Scl 75/100 Th/To Fibrillarin (U3RNP) Ku Ro52/TRIM21	5-15%	Variation in gap dependent on variables in SSc cohorts studied: • Classification criteria • Diagnostic criteria • Cross-sectional vs. Inception	[55,56]
SjS	SSA/Ro60 SSB/La	15-25%		[57,58]
IIM	tRNA synthetases* SRP HMGCR MDA-5 Mi-2 SAE TIF1γ NXP2 NT5c1A/Mup44 Ro52/TRIM21	10-15%	Variation in gap dependent on variables in IIM cohorts studied: • Classification criteria • Diagnostic criteria • Cross-sectional vs. Inception • Here IIM includes sIBM	[49,59-61]
APS	Cardiolipin β2GPI β2GP1 (domain 1) PS/PT	15-30%	Some clinicians rely on strict serological criteria (cardiolipin, β2GP1, lupus anticoagulant) in current classification criteria	[62,63]
MCTD	U1RNP	0%	By definition anti-U1RNP is a required criterion. MCTD should not be confused with the broader spectrum of overlap syndromes or UCTD	[64,65]
RA	RF ACPA CarP* PAD4*	15-20%	Combining RF and ACPA (and CarP) increases sensitivity	[66-69]

**Assays currently available as research use only*

Abbreviations: ACPA: Anti-Cyclic Citrullinated Peptides; APS: Antiphospholipid Syndrome; β2GPI: Beta 2 Glycoprotein 1; CarP: Carbamylated Peptides; dsDNA: double stranded DNA; HMGCR: Hydroxy Methyl Co-Reductase; IIM: Idiopathic Inflammatory Myopathies; Ku: DNA Phosphokinase; MCTD: Mixed Connective Tissue Disease; MDA-5: Melanoma Differentiation-Associated Protein 5; Mi-2: A Component of the Nucleosome Remodeling-Deacetylase Complex; Mup44: Skeletal Muscle Antigen; NT5c1A cytosolic 5': Nucleotidase 1A; NXP2: Nuclear Matrix Protein 2; PAD: Protein Arginine Deiminase; PCNA: Proliferating Cell Nuclear Antigen; RNP: Ribonucleoprotein; SAE: Sumo Activating Enzyme 1; sIBM: Sporadic Inclusion Body Myositis; SSA: Sjögren's Syndrome Antigen A; SSB: Sjögren's Syndrome Antigen B; SjS: Sjögren's Syndrome: SLE: Systemic Lupus Erythematosus; SRP: Signal Recognition Particle; SSc: Systemic Sclerosis; TIF1γ: Transcription Intermediary Factor 1-Gamma; tRNA: transfer RNA; UCTD: Undifferentiated Connective Tissue Disease

One of the claims that HEp-2 should be the 'gold standard' was based on the notion that the HEp-2 cell represented a "mini-array" of >100 target autoantigens [10]. However, this claim failed to recognize that while this is theoretically correct, in practice many well know targets (e.g. Ro52/TRIM21, Ro60/SSA, ribosomal P proteins, Jo-1, to name a few) do not give consistent IIF patterns on HEp-2 substrates [7,8]. This gap between theory and practice is reminiscent of an adage attributed to Yogi Berra, "In theory there is no difference between practice and theory; in practice there is" [13]. Hence, the notion that HEp-2 cells are a multi-analyte array (MAA) and that IIF is the preferred test platform became engrained in clinician's thinking and even prompted some autoantibody test kit manufacturers to roll back newer diagnostic platforms and ramp up production and marketing of HEp-2 IIF kits. This had an overall beneficial effect because it challenged the diagnostic industry to close the gap between high-throughput SPA ANA screening and HEp-2 IIF. Some studies now report that SPA ANA screening assays are equivalent or superior to the HEp-2 IIF test and are also a cost-effective alternative [14-16]. Last, the 'gold standard' proclamation marked the advent of automated ANA IIF technologies, which further closed technical and subjective interpretation gaps in ANA IIF testing [1,17].

Another factor widening the spectrum of ANA and autoantibody testing is a concerted move to preventive medicine and precision health (PH). Until recently, it has been assumed that the primary use of ANA and autoantibody testing is to support the diagnosis of a SAID with 'intent to treat' [7] and as criteria for entry into clinical trials [18,19]. However, an emerging evidence-based approach to very early SAID identification and 'case finding' is focusing on 'intent to prevent' morbidity and mortality associated with SAIDs [7,20,21]. In very early SAIDs, signs and symptoms do not always point to a single 'high pretest probability' disease, necessitating a paradigm shift in diagnostics where the focus is on testing individuals based on evidence-based risk factors and the earliest signs and symptoms (lower pre-test probability) of SAIDs. Real-time MAA data on patients starting at the earliest onset of disease has the potential to guide further investigations (biopsy, imaging, etc.), referrals to appropriate specialists, and serve as a guide to treatment, predicting disease flares and confirming remissions. One of the anticipated benefits of an earlier and accurate diagnosis is decreasing health care expenditures [7,21].

Standardization gaps

A significant limitation of ANA IIF testing is the tremendous gap in standardizing the test; an issue that persists almost half a century after the adoption of this assay. Despite numerous studies and analyses, there is no universally accepted screening serum dilution (for adults or children) [22], different manufacturers use different secondary antibodies, the cells are grown and fixed with differing protocols, the assay is semi-quantitative at best and the interpretation of various IIF patterns is highly observer dependent [19,23,24]. Part of the challenge is that despite attempts at protocol standardization and regulatory protocols (i.e., 510K approval by the Food and Drug Administration USA), diagnostic laboratories often do "what is right in their own eyes" [25,26]. Thankfully, as mentioned above, advances are being made in the performance of the IIF test through automated robotics and digital image analytics accompanied by progress in standardization of the nomenclature of ANA IIF patterns [12,27]. The lingering, and seemingly unresolvable, limitations of IIF testing on HEp-2 cells portend a continuing replacement of this test with SPA and MAA that can outperform it. Indeed, akin to the proposed inverted 'pyramid' of reflex testing in the ANCA testing [28], there is a sense that ANA screening should follow suit and broad-spectrum screening tests be replaced by Multi-Analyte Arrays with Algorithmic Analyses (MAAAAs) [29]. The MAA component of technology platforms are well developed and increasingly available but a gap to be closed is the last AA (algorithmic analyses) using artificial intelligence to link big data to clinical care pathways [30,31].

Assay performance and 'seronegative' gaps

There should be no assumptions that a move to newer, high-throughput technologies is nirvana [32]. Indeed, some old challenges will persist and new challenges will arise. Inter-manufacturer and inter-laboratory variability will continue to be a challenge, although standardization appears to be more easily attainable because purified components providing quantitative results are typically used in newer MAA platforms. Hence, a goal of standardized performance could be based on international reference standard sera and the assignment of results in 'international units" [33,34]. This means that for every antigen in a MAA, an internal reference standard should be required, an important technical gap that needs to be and can be addressed.

Despite the discovery and description of numerous autoantibody targets, a seronegative gap persists for many SAIDs **(Table 2)**. It seems ironic that after more than 50 years of research that a serological gap persists despite, for example, more than 180 targets of autoantibodies being described in SLE [35], only a handful are used in diagnostic assays **(Table 2)**. Akin to other discoveries, many targets perish in the innovation "valley of death" [36,37]. The reasons for this are not well studied for autoantibodies but the selection of certain autoantibody targets and the rejection of others is dependent on their ability to be independently validated and meet SMAARTT criteria: Specificity for disease balanced by acceptable Sensitivity, are they Measurable in conventional diagnostic platforms, are they Actionable or associated with a clear clinical Advantage or outcome (i.e., predictive, prognostic), are they Realistic, Timely and Titratable [38]. Therefore, as SAID-specific

MAAAAs are developed, it will also be important to continue to fill the "seronegative" gap in these conditions. Fortunately, the search for novel SARD autoantibody targets that close the seronegative gap is a productive academic and industry enterprise with novel candidates continuously reported. In addition, rescuing targets that have perished in 'death valley' and their incorporation into new MAAAAs may be a rewarding data mining exercise.

Nomenclature gaps

It is well known that many of the autoantibody targets in SAID are not restricted to the nucleus [12]. Hence, the term ANA is technically inaccurate and misleading because many of the SAID autoantibody targets are in the cytoplasm and/or directed to mitotic cells [39,40]. However, proposals to change the terminology from ANA to anti-cellular antibodies (ACA) have been met with resistance [12].

Another concern is persisting misunderstanding and lack of attention to well-defined autoantibody systems in SAID. For example, current literature is replete with misnomers such as confusing the SSA/Ro60 antigen system with the Ro52/TRIM21 system. This in part has been fostered by diagnostic companies who persist in combining these two antigens into a single assay. Although once thought to be part of the same subcellular macromolecule and linked to the diagnosis of SjS [41], the molecular evidence and clinical correlations no longer support either claim [42]. Indeed, anti-SSA/Ro60 and SSB/La are key autoantibody biomarkers for SjS, but anti-SSA/Ro60 is aslo seen in a broad spectrum of SAID and related conditions [42], such as subacute cutaneous and neonatal lupus [43-46]. And, while anti-SSA/Ro60 can co-exist with anti-Ro52/TRIM21, the latter is seen in an even wider spectrum of SAID [47], is the second most common antibody detected in SSc sera [48] and in IIM is particularly common in the anti-Jo-1 subset of anti-synthetase syndrome [49]. These and a number of other compelling reasons should bring to a close the notion that somehow SSA/Ro60 and Ro52/TRIM21 can be tested together or that they fit together into a clear-cut clinical paradigm.

Classification criteria closing gaps

Curiously, despite the limitations of the ANA IIF test, it continues to be a key criterion in the classification of some of SAIDs, especially SLE. The most recently revised SLE classification criteria supported by the American College of Rheumatology (ACR) and the European League Against Rheumatism (EULAR) uses the ANA IIF as a required entry criterion [50,51], apparently taking into consideration that the lack of specificity of the ANA test for SLE is counter-balanced by the other weighted clinical and serological findings. Two issues regarding the new ACR/EULAR criteria should be noted. First, analysis of the comparative performance of the three prevalent SLE classification criteria, the Revised ACR Criteria [52] and the SLICC Criteria [53], suggests that the newer EULAR/ACR criteria are a step in the right direction **(Table 3)** [51]. Second, with respect to the ANA HEp-2 IIF requirement, "an equivalent" assay is a permissible option. This criterion may create misunderstanding because by not defining the characteristics of an "equivalent" test, it has no technical comparator definition except the assumption that this equivalence will relate to sensitivity and specificity of the ANA at a titer of ≥ 1/80. This may be a moot point because, as discussed above, there is an apparent progressive move to high-throughput ANA testing by MAA that is not only equivalent to but exceeds the performance of the ANA IIF test.

Table 3. Comparative sensitivity and specificity of SLE classification criteria* [51].

	ACR 1997 [52]	SLICC 2012 [53]	EULAR/ACR [50,69]
Derivation			
Sensitivity	0.85	0.97	0.98
Specificity	0.95	0.90	0.96
Combined	1.79	1.87	1.94
Validation			
Sensitivity	0.83	0.97	0.96
Specificity	0.93	0.84	0.93
Combined	1.76	1.80	1.90

** Rounded to two decimal points*
Abbreviations: ACR: American College of Rheumatology; EULAR: European League Against Rheumatism; SLICC: SLE International Collaborating Clinics

CONCLUSION

Gaps in autoantibody testing, especially the anti-nuclear antibody (ANA) IIF test are largely driven by the clinical approaches that focus on screening for SAIDs in patients with low pre-test probability, a wide spectrum of clinicians who order the tests and continuous evolution of diagnostic test platforms. Some gaps, such as nomenclature persist despite knowledge available to fill them, while other gaps require additional effort and international collaboration.

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