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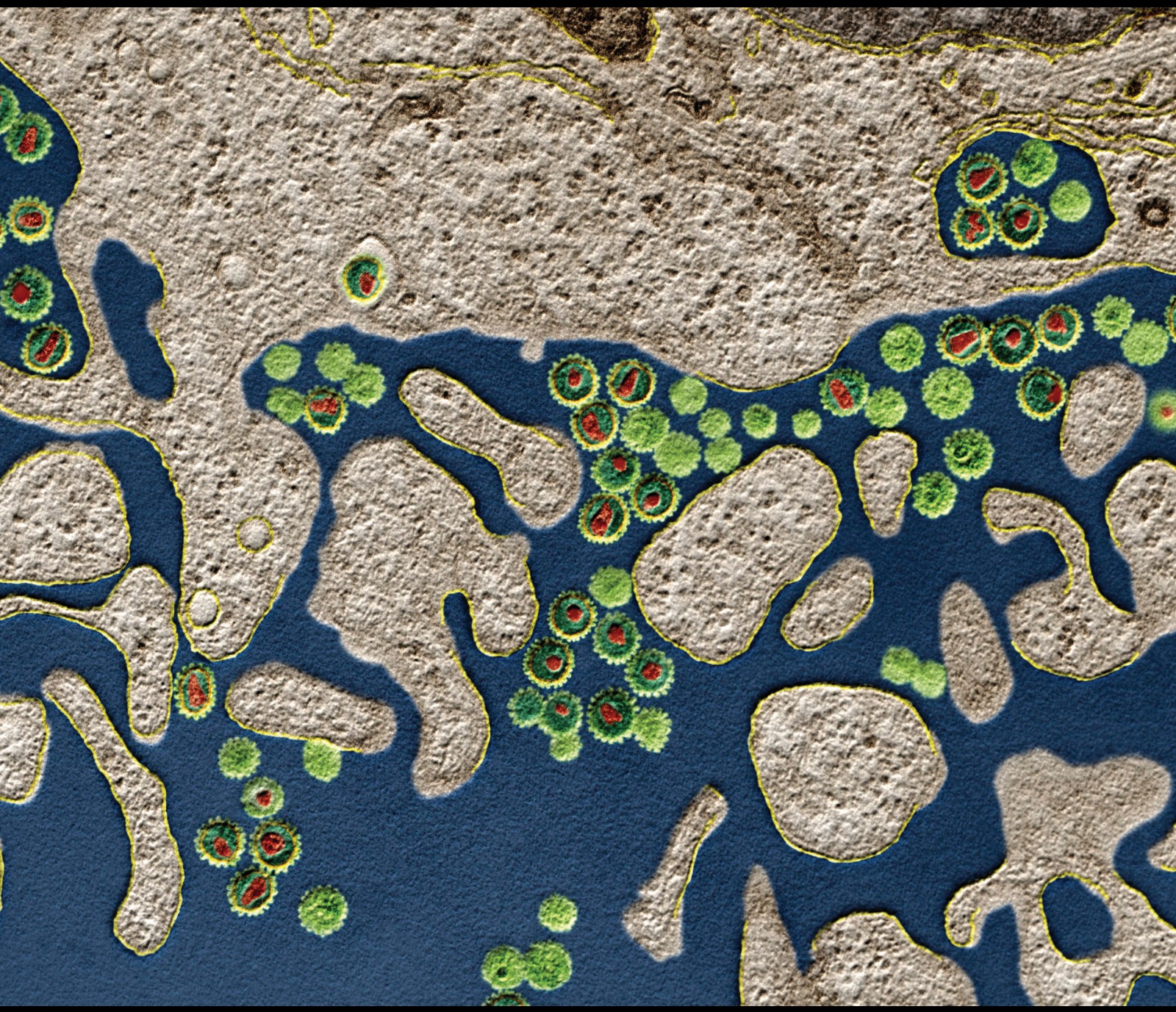
Autoantibodies in Systemic Autoimmune Disorders

Guest Editors: Michael Mahler, Silvia Pierangeli, Pier-Luigi Meroni, and Marvin J. Fritzler



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Editorial

Autoantibodies in Systemic Autoimmune Disorders

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Over the past decade, there has been increased awareness and understanding of autoimmune diseases and many conditions once considered idiopathic have now been attributed to autoimmune phenomena. To meet these challenges, novel disease modifying drugs have been developed for some autoimmune diseases allowing for earlier and more effective intervention and thus the prevention of irreversible damage of certain organs. Consequently, there is a growing need for novel or improved biomarkers to enable early and precise diagnosis of these conditions. This special issue focusses on autoantibodies in systemic autoimmune diseases and includes a collection of 16 manuscripts (including original articles and reviews) reporting on various aspects of serum autoantibody research ranging from the identification of novel disease-related autoantigens to the clinical significance of autoantibodies.

As one example of the expanding spectrum of autoimmune diseases, T. Takeda and Y. Sakurai provide evidence that acquired hemophilia has clinical and serological features of an autoimmune disorder. Several articles discuss various aspects of the detection of antinuclear antibodies (ANA), which are a hallmark in the diagnosis of systemic autoimmune rheumatic diseases (SARD) [1]. In a comprehensive review article on ANA, advances in ANA testing and persistent challenges are discussed. One of the recent advances in ANA testing is the development and utilization of various digital imaging systems. Although the systems from various manufacturers differ in one way or another, most of them demonstrate comparable functions and performance characteristics. One of the systems, NOVA View, was used by S. S. Copple et al. to demonstrate that the indirect immunofluorescence (IIF) results provided by this system are comparable to manual reading, a feature that holds promise to overcome the subjectivity of IIF and the need for a dark room to read the slides. Additional benefits of digital imaging systems are the facilitation and standardization of diagnostic procedures, as well as creating a permanent archival record of the results. T. Y. Avery et al. investigated the prevalence and titers of ANA by IIF in primary, secondary, and tertiary care settings and showed that both prevalence and ANA titers increase from primary to tertiary care. The use of alternative methods for ANA testing has been discussed. Interestingly, very high prevalence of ANA was reported by M. Segni et al. in children with autoimmune thyroid disease.

Besides ANA testing, the subdifferentiation of autoantibody reactivity to extractable nuclear antigens (ENA) is an important part of the diagnosis of patients with SARD. Several novel methods have been developed over the years and careful validation is mandatory. M. Infantino et al. compared different systems for the detection of SS-A/Ro60, Ro52/TRIM21, and SS-B/La autoantibodies in human sera. Overall, the results showed good concordance. Historically described in patients with rheumatoid arthritis (RA), anti-hnRNP B1 antibodies, also known as anti-Ra33, were studied by A. Maslyanskiy et al. in patients with RA and systemic sclerosis (SSc). The results indicate that anti-Ra33 positive patients represent a distinct clinical RA phenotype.

Three articles focused on myositis specific autoantibodies and their detection. Y. Muro et al. described a new cDNA protocol to facilitate the development of ELISA systems for

the detection of myositis associated antibodies (e.g., Mi-2, MDA5, NXP-2, TIF1-α, and TIF1-γ). A second article that focused on myositis studied anti-MDA5 antibodies which have been reported in patients with rapidly progressing interstitial lung disease and therefore are considered as a prognostic biomarker. C. Juárez et al. described anti-MDA5 antibodies in a Mediterranean cohort of IIM patients. The anti-MDA5 positive patients presented rapidly progressive interstitial lung disease (RP-ILD) with a cumulative survival rate that was significantly lower than that in the remainder of the series ($P < 0.05$).

In 2010, autoantibodies to 3-hydroxy-3-methylglutaryl-coenzyme A reductase (HMGCR) were described in patients with immune mediated necrotizing myopathies (IMNM). From an immunopathogenic perspective, these findings were of special interest because HMGCR is the primary molecular target of statin therapies. Commercial immunoassays have become available for routine diagnostics to detect anti-HMGCR antibodies and in the study by L. Musset et al., different research methods demonstrated excellent agreement, which is important in light of the challenges to standardize autoantibody assays.

Antiphospholipid syndrome (APS) is an autoimmune disorder characterized by recurrent arterial or venous thrombosis and miscarriages. Additional nonclassification criteria have been also reported such as heart valve disease, livedo reticularis, thrombocytopenia, nephropathy, and neurological manifestations other than ischemic events [2]. Thrombotic events are mediated by antiphospholipid antibodies (aPL), such as anticardiolipin antibodies (aCL) and/or anti-β2 glycoprotein I (β2GPI) and/or lupus anticoagulant (LAC) [3]. APS can occur in isolation (primary APS) or in association with other autoimmune diseases such as systemic lupus erythematosus (SLE) (secondary APS). Recent studies have described patients with clinical evidence of APS who did not fulfill the serological criteria for the disease. Some patients with APS are negative for the classification criteria biomarkers but have other autoantibodies such as IgA anti-β2GPI antibodies. Several studies also reported patients with clinically overt disease but without any autoantibodies that bind t anionic PL, β2GPI, or prothrombin, an important subset of APS referred to as "seronegative APS."

A significant subpopulation of RA patients develop extra-articular manifestations (EAM) of their disease. Since some of the EAM can be life-threatening, biomarkers are needed to aid in the diagnosis of EAM. In the study by J. I. Gamez-Nava et al., EAM were associated with disease duration, DAS28, and higher HAQ-DI score but not with serum levels of anti-CCP and anti-MCV antibodies.

Two articles focused on autoantibodies in systemic lupus erythematosus (SLE). J. Li et al. reported on the association of autoantibodies with clinical manifestations in a large Chinese SLE cohort of patients. The second manuscript investigated the association between antilymphocyte antibodies and disease activity as well as lymphopenia. Last, neonatal lupus syndrome is characterized by bradycardia, heart block, and a variety of other findings including autoantibodies to SSA/Ro60 and a cutaneous eruption in the newborn resembling subacute cutaneous lupus. In a study expanding the paradigm to second trimester pregnancies, A. Wacker-Gussmann et al. reported on atrioventricular delay as measured by fetal magnetocardiography.

This special issue provides insight and gives the reader a sense of some of the advancements made in autoimmunity and autoantibody research over a short time interval.

Acknowledgment

Sadly, Silvia Pierangeli [4], one of the most respected, prolific, and well-liked investigators in the field of antiphospholipid antibody/antiphospholipid syndrome research passed away during the genesis of this special issue. We acknowledge with deep gratitude Silvia's great contributions to APS research and to this special issue.

Michael Mahler
Silvia Pierangeli
Pier-Luigi Meroni
Marvin J. Fritzler

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Research Article

Anti-hnRNP B1 (RA33) Autoantibodies Are Associated with the Clinical Phenotype in Russian Patients with Rheumatoid Arthritis and Systemic Sclerosis

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Heterogeneous nuclear ribonucleoproteins (hnRNPs) are potent autoantigenic targets in systemic autoimmune rheumatic diseases (SARD). Loss of tolerance to the RA33 complex consisting of hnRNP A2 and its alternatively spliced variants B1 and B2 has been the interest of rheumatologists. A novel ELISA for the detection of anti-hnRNP B1 autoantibodies has been developed to investigate the prevalence thereof in 397 patients with SARD, including patients with rheumatoid arthritis (RA), spondyloarthropathy (SPA), juvenile chronic arthritis, systemic lupus erythematosus (SLE), systemic sclerosis (SSc), and Sjögren's syndrome (SS), in comparison to 174 controls. Anti-hnRNP B1 autoantibodies were significantly more prevalent in patients with SARD than controls (47/397, 11.8% versus 2/174, 1.1%; $P < 0.001$). In particular, anti-hnRNP B1 were found more frequently in the disease cohorts than in the controls and were present in 24/165 (14.5%) patients with RA, 6/58 (10.3%) SPA, 11/65 (16.9%) SSc, and 4/50 (8.0%) SLE. In RA patients, anti-hnRNP B1 autoantibodies correlated significantly with C-reactive protein levels and erythrocyte sedimentation rate, while in patients with SSc it was associated with features of arterial wall stiffness and presence of hypertension. Anti-hnRNP B1 autoantibodies occur in SARD and seem to be correlated with distinct clinical characteristics in patients with RA and SSc.

1. Introduction

Heterogeneous nuclear ribonucleoproteins (hnRNPs) are nucleoplasmic molecules interacting with premessenger ribonucleic acid (pre-mRNA) and partake in the processing thereof [1]. In general, hnRNPs contain at least one RNA recognition motif representing the RNA-binding domain. Furthermore, they can play a role in various other important cellular mechanisms like DNA repair, telomere elongation, chromatin remodelling, and translocation, as well as nuclear-cytoplasmic shuttling, translation, and regulation of proteins. Loss of immunological tolerance to hnRNP has been reported in several systemic autoimmune rheumatic diseases (SARD) [2]. Hitherto, 30 major hnRNPs with the terminology A1

Table 1: Characteristics of individuals studied including 397 patients with systemic autoimmune rheumatic diseases and 174 controls.

Diagnosis	*n*	Gender f/m	Age median	Age IQR	DD median	DD IQR	Clinical characteristics
RA	165	102/63	54.0	47.0–60.0	0.58	0.3–5.0	Diagnosis was based on ACR/EULAR 2010 criteria, median DAS28: 4.3 (IQR 3.3–5.2)
SPA	58	17/41	37.0	32.0–50.0	7.0	4.5–12.0	52 pts with ankylosing spondylitis according to the New York criteria and 6 pts with axial spondyloarthritis according to the ASAS criteria 2010
JCA	42	26/16	10.6	6.9–15.4	4.4	1.0–9.9	12 pts with polyarticular disease, 24 pts with oligoarticular disease, 6 pts with systemic disease
SLE	50	47/3	36.0	27.0–45.0	5.0	3.0–12.0	Diagnosis was based on ACR 1997 Revisited criteria, median SLEDAI 4.0 (IQR 2.0–8.0)
SSc	65	62/3	53.0	42.0–60.0	5.0	3.0–9.0	Diagnosis was based on ACR (ARA) criteria 1980 Rodnan skin involvement score: median 16 (IQR 9–22), 31 pts with diffuse scleroderma, 27 pts with limited scleroderma, 7 pts with overlap syndrome
SS	17	17/0	62.5	55.0–66.0	5.0	4.0–11.0	Diagnosis was based on American-European classification criteria, median ESSPRI: 5.3 (IQR 3.8–7.0)
HYI (cardiology control)	52	33/19	52.0	44.0–55.0			Clinically observed for absence of rheumatic diseases
BD	122	83/39	33.0	29.5–42.0			

ACR: American College of Rheumatology; DAS28: disease activity score of 28 joints; DD: disease duration; ESSPRI: The EULAR Sjögren's Syndrome Patient Reported Index; EULAR: European League Against Rheumatism; HYI: hyperlipidemic individuals with high Framingham cardiovascular score; JCR: juvenile chronic arthritis; *n*: number; pts: patients; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SLEDAI: systemic lupus erythematous disease activity index; SPA: spondyloarthropathy; SS: Sjögren's syndrome; SSc: systemic sclerosis.

through U have been described. Of them particularly hnRNP A1, A2, B, C, H, I, and R could be demonstrated as autoantigenic targets in SARD [3].

Autoreactivity to the RA33 complex mainly consisting of autoantibodies to hnRNP A2 and its alternatively spliced variants B1 and B2 has been demonstrated in patients with rheumatoid arthritis (RA) as early as 1989 [4]. Thus, the respective autoantibody was referred to as anti-RA33 because of its reaction with a 33 kDa antigen by immunoblotting employing nuclear extracts from HeLa cells. Apart from immunoblotting, enzyme-linked immunosorbent assay (ELISA) has been employed mainly to test for anti-RA33, but experimental testing has led to inconsistent results amongst studies. Nevertheless, several reports revealed a prevalence of about 30% for anti-B1/A2 hnRNP autoantibodies in patients with RA [5]. However, those autoantibodies have been also found in patients with systemic lupus erythematosus (SLE) and other SARD [6, 7]. Such data challenged the original notion that anti-RA33 autoantibodies are highly specific for RA [7]. Along with other RA-specific autoantibodies, such as rheumatoid factor (RF) and anticitrullinated peptide/protein antibodies (ACPA), these antibodies are of interest to rheumatologists as they appear to be present in early disease states, especially in RF-negative patients [8, 9]. Furthermore, they are associated with relatively mild and nonerosive disease in the absence of high-titer RF and ACPA such as anticitrullinated cyclic peptide (CCP) antibodies [8]. Recently, several anti-hnRNP autoantibodies have been investigated in patients with SARD [10]. Such a meticulous assessment concluded that the most prevalent anti-RA33 antibody by ELISA is directed against hnRNP B1.

The aim of the present study was to develop a novel ELISA detecting anti-hnRNP B1 autoantibodies and to investigate their prevalence in a Russian cohort of patients with RA and other SARD, as well as controls. As these autoantibodies are directed against a complex with pleiotropic functions, we speculated that autoreactivity against hnRNP B1 could bear pathogenic significance and it is of clinical relevance, stratifying patients according to distinct clinical phenotypes. Thus, we also attempted to correlate the occurrence of anti-hnRNP B1 autoantibodies with disease-related clinical manifestations.

2. Patients and Methods

2.1. Patients. In total, 397 patients with SARD and 174 controls were enrolled in the study. Characteristics of patients and controls are outlined in Table 1. Patients with SARD consisted of 165 patients with RA, 58 patients with spondyloarthropathy (SPA), 42 patients with juvenile chronic arthritis (JCA), 50 patients with SLE, 65 patients with systemic sclerosis (SSc), and 17 patients with Sjögren's syndrome (SS). Diagnosis of SARD had been established based on typical clinical, biochemical, histological, and serological features according to the criteria of the respective classification criteria of each SARD. Controls consisted of 52 hyperlipidemic donors in whom there was no current evidence or past

medical history of SARD. Furthermore, 122 blood donors were included in the control group (Table 1).

The study was approved by the ethics committee of Almasov's Centre, St. Petersburg, vote number 12421, May 2012. Aliquots of the sera stored at −20°C were used for the study of antibody reactivity.

2.2. Assessment of Vascular Stiffness. Measurement of vascular stiffness by pulse wave velocity (PWV) and augmentation index (AI) was performed using applanation tonometry with the SphygmoCor system (AtCor Medical Pty Ltd., Sydney, Australia). Briefly, PWV and AI adjusted to a heart rate of 75 beats per minute were registered in subgroups of patients with late RA, SSs, SPA, and HYI as cardiology controls. Pulse wave velocity was assessed in patients after 15 minutes of rest in a sitting position. Measurements were done consequentially above carotid and femoral arteries during 10 seconds with simultaneous registration of electrocardiography, which was used to determine the moment of heart contraction. Distance of pulse wave propagation from carotid arteries to femoral arteries was measured directly and divided by the time of wave propagation to calculate pulse wave velocity. The reference ranges for PWV were defined as less than 10 meters per second.

Augmentation index was calculated from the difference between first and second wave of pulse pressure expressed as a percentage of pulse pressure. Pulse pressure was registered with pinpoint probe over ulnar artery during 10 seconds.

2.3. Detection of Anti-hnRNP B1 Autoantibodies by ELISA. Anti-hnRNP B1 IgG was assessed in serum samples of patients and controls by an ELISA. This assay employs recombinant human hnRNP B1 expressed in *E. coli* (in.vent DIAGNOSTICA GmbH, Hennigsdorf, Germany). Briefly, hnRNP B1 at a concentration of 5 mg/L was coated onto the solid phase of Maxisorb microtiter plates (Thermo Scientific Inc./Nunc, Germany) in bicarbonate buffer, pH 9.5, at 4°C for 26 h. After blocking with 0.05 mol/L Tris-HCl and 1% bovine serum albumin (TrisBSA, pH 7.4) at room temperature (RT) for 1 h, serum samples diluted 1 : 100 in TrisBSA were incubated at RT for 1 h and washed. Horseradish peroxidase-conjugated anti-human IgG was added and developed with ready-to-use H_2O_2/TMB substrate. The reaction was stopped with 0.25 mol/L sulphuric acid after 15 min. The optical density (OD) of the samples was read using a microplate reader (BioTek Instruments Inc., Winooski, USA) at a wavelength of 450 nm against 620 nm and results were expressed as arbitrary units (U/mL). The cut-off for positivity at 10 U/mL determined by receiver operating characteristics curve analysis was used. The functional assay sensitivity [11] representing the lowest antibody concentration with a coefficient of variation smaller than 20% was determined at 6.4 U/mL. The intra- and interassay variances were determined at 4% and 6%, respectively, employing a serum with a concentration of 12.0 U/mL anti-hnRNP B1 antibody. Testing 299 sera of patients suffering from RA, anti-hnRNP B1 antibody analysis by the novel ELISA was correlated with anti-hnRNP A2 (RA33) antibody detection by a commercially available ELISA (HUMAN, Wiesbaden, Germany) (see Supplementary Figure 1 in Supplementary Material available online at http://dx.doi.org/10.1155/2014/516593). There was a weak, yet significant, correlation between both anti-hnRNP antibody assays (Spearman's rho = 0.209, 95% confidential interval [CI]: 0.098–0.315; $P < 0.001$). In contrast to the commercially available assay, the novel ELISA demonstrated no significant difference in anti-hnRNP antibody levels testing fresh and unthawed aliquots of long-term stored sera.

To confirm the specificity of anti-hnRNP B1 antibody detection by the novel ELISA, 5 sera of patients suffering from rheumatoid arthritis and systemic sclerosis each demonstrating anti-hnRNP B1 IgG positivity were tested by immunoblot employing the recombinant hnRNP B1 polypeptide. All 10 sera demonstrated a clear positive reaction to hnRNP B1 blotted onto a nitrocellulose membrane (Supplementary Figure 2).

2.4. Detection of RA-Specific Autoantibodies. Rheumatoid factor was determined with Tina-Quant immunoturbidimetry assay (Roche Diagnostics/Roche Deutschland Holding GmbH, Penzberg, Germany) and results were expressed in IU/mL. Concentrations over 15 IU/mL were scored positive. Anti-CCP IgG was determined by ELISA according to the instructions of the manufacturer (Euroimmun AG, Lübeck, Germany). The OD was read in a microplate reader at 450 nm and results were expressed as relative units (RU/mL). The cut-off for positivity at 5 RU/mL in accordance with the recommendations of the manufacturer was used for these assays.

2.5. Statistical Analysis. A Kolmogorov-Smirnov test was used to analyse the data for normality. The measured values were expressed as medians with 95% CI. The two-tailed, nonparametric Mann-Whitney and Kruskal-Wallis tests were used to test for statistically significant differences of independent samples in 2 or more groups, respectively. The nonparametric Wilcoxon test was employed to test paired samples.

Spearman's rank correlation test was applied for within-group comparison. Comparison of prevalence rates between groups was performed by two-tailed Fisher's exact test. P values less than 0.05 were considered significant. Calculations were performed using Medcalc statistical software (Medcalc, Mariakerke, Belgium).

3. Results

3.1. Anti-hnRNP1 B1 Autoantibodies in SARD. Elevated anti-hnRNP B1 autoantibodies have been found to be significantly more prevalent in patients with SARD (47/397, 11.8%) in comparison with controls (2/174, 1.1%) ($P < 0.001$, Table 2). Amongst SARD patients, those with RA (24/165, 14.5%), SPA (6/58, 10.3%), SSc (11/65, 16.9%), and SLE (4/50, 8.0%) demonstrated significantly higher prevalences of anti-hnRNP 1 autoantibodies compared to controls ($P < 0.001$, $P = 0.004$, $P < 0.001$, and $P = 0.023$, resp.).

Table 2: Prevalence of anti-hnRNP B1 autoantibodies in sera of 397 patients with systemic autoimmune rheumatic diseases and 174 controls.

	RA $n = 165$	SPA $n = 58$	JCA $n = 42$	SSc $n = 65$	SLE $n = 50$	SS $n = 17$	SARD $n = 397$	HYI $n = 52$	BD $n = 122$	Controls $n = 174$
Positives, >10 U/mL	24 (14.5%)	6 (10.3%)	1 (2.4%)	11 (16.9%)	4 (8.0%)	1 (5.9%)	47 (11.8%)	0 (0.0%)	2 (1.6%)	2 (1.1%)

HYI: hyperlipidemic individuals with high Framingham cardiovascular score; JCR: juvenile chronic arthritis; *n*: number; RA: rheumatoid arthritis; SLE: systemic lupus erythematosus; SPA: spondyloarthropathy; SS: Sjögren's syndrome; SSc: systemic sclerosis.

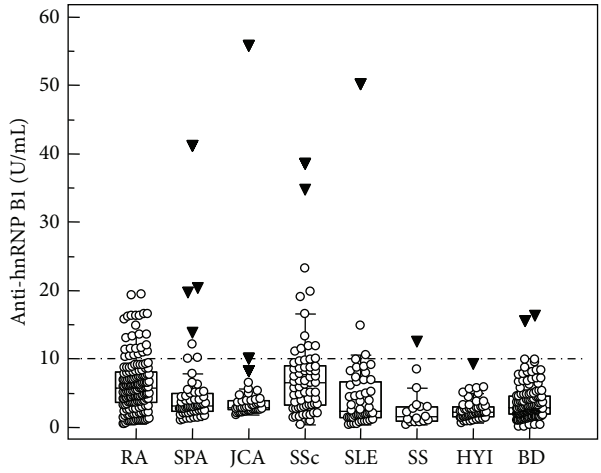

Figure 1: Anti-hnRNP B1 IgG levels in 165 patients (pts) with rheumatoid arthritis (RA), 58 pts with spondyloarthropathy (SPA), 42 pts with juvenile chronic arthritis (JVA), 65 pts with systemic sclerosis (SSc), 50 pts with systemic lupus erythematosus (SLE), 17 pts with Sjögren's syndrome (SS), 52 controls with hyperlipidemia, and 112 blood donors (BD) detected by enzyme-linked immunosorbent assay (ELISA). (Data are displayed as U/mL in Box-and-Whisker plots with far-out values, defined as values that are smaller than the lower quartile minus 3 times the interquartile range or larger than the upper quartile plus 3 times the interquartile range, displayed as solid triangles.)

Anti-hnRNP B1 autoantibody levels differed significantly amongst the 397 patients with SARD and the 174 controls (ANOVA, Kruskal Wallis test, $P < 0.001$) (Figure 1). Amongst patients with SARD, the highest anti-hnRNP B1 autoantibody concentrations were found in patients with RA (median: 5.8, interquartile range [IQR]: 3.6–8.1) and SSc (median: 6.5, IQR: 3.2–9.0) which differed significantly from the other patient groups (SPA, JCA, SLE, and SS, $P < 0.001$, resp.) and controls (HLI and BD, $P < 0.001$, resp.). However, patients with RA and SSc did not reveal significantly different anti-hnRNP B1 autoantibody levels ($P > 0.05$).

3.2. Anti-hnRNP1 B1 Autoantibodies in RA. Rheumatoid arthritis-specific RF showed a prevalence of 118/165 (71.5%) in serum samples of 165 patients with RA, whereas antibodies against CCP were elevated in 126/165 (76.4%) sera thereof (Table 3). There was no correlation between anti-hnRNP autoantibody levels and those of RF and anti-CCP antibody according to rank correlation analysis (Table 4). Furthermore, neither in RF-positive compared with RF-negative patients nor in anti-CCP antibody positives against anti-CCP antibody negatives were anti-hnRNP B1 autoantibodies significantly different ($P > 0.05$, resp.). There was also no significant difference in RF and/or anti-CCP antibody positives compared with the respective negatives ($P > 0.05$).

Interestingly, a significant negative correlation of anti-hnRNP B1 autoantibody with disease duration could be established hinting at an early occurrence thereof in RA patients. However, there was no significant difference of anti-hnRNP autoantibody levels as well as prevalence in early (disease duration of less than 12 months, $n = 102$) and established RA ($P > 0.05$). Although anti-hnRNP B1 autoantibodies correlated significantly with C-reactive protein (CRP) levels, erythrocyte sedimentation rate (ESR), and joint space narrowing of hands, there was no significant association with the disease activity score of 28 joints (DAS28) in the patients with RA.

3.3. Anti-hnRNP1 B1 Autoantibodies in SSc. To investigate the association with clinical phenotype of patients with SSc, clinical characteristics thereof have been obtained (Table 5). In contrast to patients with RA, there was no significant correlation between anti-hnRNP B1 autoantibody and ESR, CRP levels, and duration of disease ($P > 0.05$). Furthermore, no significant correlation of autoreactivity to hnRNP B1 with fibrotic clinical manifestations in SSc such as lung and skin involvement could be established. Given the relative small number of the sera tested from patients with SSc, a safe conclusion cannot be reached and larger studies are warranted. However, like in patients with RA, there was a significantly positive correlation with the age of patients in this group. Interestingly, a significantly positive correlation with the presence of clinical manifestations including digital ulcers and esophagitis could be established ($P = 0.007$, $P = 0.016$). Notably, anti-hnRNP B1 autoantibodies demonstrated an association with hypertension and associated features such as arterial wall elasticity and pulse wave velocity (PWV) ($P = 0.009$, $P = 0.009$, and $P = 0.004$, resp.). Preliminary assessment revealed a noteworthy positive correlation of anti-hnRNP B1 autoantibodies with PWV in patients with RA ($n = 39$, Spearman's rho = 0.41; $P = 0.009$) but not in patients with SPA and HYI ($P > 0.05$, resp.).

4. Discussion

Loss of immune tolerance to components of large RNP moieties being part of spliceosomes or ribosomes seems to be characteristic for distinct SARD [2]. In particular, autoantibodies against the hnRNP complex composed of pre-mRNA

Table 3: Anti-hnRNP B1 autoantibodies in 165 patients with rheumatoid arthritis depending on the presence of rheumatoid factor and anti-CCP antibodies.

	RF		Anti-CCP		RF/anti-CCP	
	Positive ($n = 118$)	Negative ($n = 47$)	Positive ($n = 126$)	Negative ($n = 39$)	Positive ($n = 102$)	Negative ($n = 23$)
n >10 U/mL	17 (14.4%)	7 (14.9%)	17 (13.5%)	7 (17.9%)	14 (13.7%)	4 (17.4%)

CCP: citrullinated cyclic peptide; RF: rheumatoid factor.

Table 4: Correlation of anti-hnRNP B1 autoantibodies with clinical characteristics of patients with RA.

	Valid number of patients	Spearman	$t\ (N-2)$	P
Age	161	0.229447	2.97253	0.003
Duration of disease	144	−0.259660	−3.20410	0.002
ESR	156	0.197243	2.49678	0.014
CRP	157	0.259968	3.35182	0.001
DAS28	120	−0.128757	−1.41040	0.161
Smoking habit	108	−0.120022	−1.24470	0.216
Anti-CCP antibody	108	0.105416	1.09141	0.278
RF	157	0.027336	0.34046	0.734
Joint space narrowing hand	75	0.309004	2.77598	0.007
Joint space narrowing feet	74	0.212152	1.84210	0.070
Erosion feet	74	0.156521	1.34470	0.183

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; CCP: citrullinated cyclic peptide; DAS28: disease activity score of 28 joints; RF: rheumatoid factor.

Table 5: Correlation of anti-hnRNP B1 autoantibodies with clinical characteristics of patients with SSc.

	Valid number of patients	Spearman	$t\ (N-2)$	P
Age	64	0.314713	2.61071	0.012
Duration of disease	64	−0.022761	−0.179267	0.858
Hypertension presence	60	0.333848	2.69726	0.009
Duration of hypertension	54	0.352815	2.71904	0.009
Augmentation index (arterial wall elasticity)	54	0.350336	2.69725	0.009
PWV	53	0.390139	3.02593	0.004
Digital ulcers	60	−0.347099	−2.81867	0.007
Esophagitis	59	−0.312778	−2.48616	0.016
Right ventricle dimensions	58	0.271581	2.11169	0.039
Heart block	57	0.254881	1.95481	0.056
ESR	62	0.236032	1.88146	0.065
CRP	59	0.218667	1.69184	0.096

CRP: C-reactive protein; ESR: erythrocyte sedimentation rate; CCP: citrullinated cyclic peptide; PWV: pulse wave velocity.

and approximately 30 different proteins have been the interest of rheumatologists as putative serological markers in SARD [10, 12]. Autoantibodies to hnRNP A2 have been described in patients with RA and are thought to be associated with milder disease [9, 13]. These autoantibodies have been proposed for autoantibody profiling in RA serological testing, as they do not seem to correlate with RF or APCA [8, 14]. Autoantibody profiling appears to be a sensible approach in the serology of SARD supported by novel developments in the modern autoimmunity laboratory addressing the need of analyzing several autoantibodies simultaneously [8, 15–19].

A comprehensive clinical study has shown that anti-hnRNP B1 autoantibodies interacting with an alternatively spliced variant of hnRNP A2 are most prevalent in patients with SARD amongst 10 different anti-hnRNP autoantibodies detected by ELISA [10]. Nevertheless, autoreactivity to hnRNP A2 does not appear to be different to that against its alternatively spliced variant B1 [7]. Thus, the present clinical study investigated anti-hnRNP B1 autoantibody levels in 397 Russian patients with SARD and 174 controls. Significantly higher anti-hnRNP B1 autoantibody prevalences and levels were found in patients with RA and SSc. Interestingly, an overlap syndrome of RA and limited SSc has been described, which was characterized by an incomplete CREST syndrome and cross-reactivity of anticentromere with anti-hnRNP B1 autoantibodies [20, 21]. Recently, an association

of anti-hnRNP autoantibodies with erosive arthritis has been described in patients with SSc [22]. Thus, anti-hnRNP autoantibody might become a nonspecific but useful marker for joint involvement in SSc patients and identify SSc patients prone to develop joint damage. In general, the radiological articular manifestations in SSc are less severe compared to those noted in patients with RA [23]. We did not find a significant correlation of articular manifestations with anti-hnRNP B1 autoantibody levels in patients with SSc.

However, in contrast to Op de Beéck et al., Russian patients with SARD demonstrated a lower prevalence of anti-hnRNP autoantibodies in patients with SARD in general [10]. In particular, Russian patients with SS and SLE showed a comparatively low prevalence (5.9% *versus* 44.1% and 8.0% *versus* 37.1% in [10], resp.). These differences could be due to the different patient characteristics of the respective cohorts or different assay performance of the ELISA employed for autoantibody testing. Indeed, detection of autoantibodies to hnRNP seems to require the preservation of conformational epitopes which can be influenced by the recombinant expression system and coating procedure for ELISA-solid phases utilized as demonstrated for other autoantigenic targets as well [24, 25]. Furthermore, Russian patients with RA can demonstrate different prevalences of RA-specific autoantibodies than reported in studies with Caucasian patients [26].

As a matter of fact, hnRNPs have important cellular functions and their respective autoantibodies could alter their functional properties [1, 3, 27]. This has led us to speculate that, in addition to their diagnostic relevance, anti-hnRNP autoantibodies may bear pathogenic potential. Thus, we attempted to correlate the loss of tolerance to hnRNP B1 with clinical characteristics in Russian patients with RA and SSc. In this study, anti-hnRNP B1 autoantibodies were not associated with disease activity or erosions in patients with RA. These findings support the assumption that anti-hnRNP antibodies are more frequent in RA patients with mild disease compared to those with more active disease [8, 9]. Interestingly, there was a positive correlation with clinical manifestations of SSc such as the occurrence of esophagitis and digital ulcers. Furthermore, an association with hypertension and arterial wall elasticity, as well as PWV, that is, features of arterial stiffness, could be established. Arterial wall stiffness is recommended as a risk factor for cardiovascular events in patients with arterial hypertension by the European Network for Non-Invasive Investigation of Large Arteries [28]. It needs to be noted that there is accumulating evidence of increased arterial stiffness in patients with SSc [29, 30]. Our preliminary results revealed a positive correlation of anti-hnRNP B1 autoantibody with PWV in RA patients, but this finding requires external validation in larger cohorts. Nevertheless, such a correlation was not seen in hyperlipidemic patients with an increased risk for atherosclerosis and arterial stiffness. To the best of our knowledge, this is the first report of a significant association of anti-hnRNP B1 autoantibodies with hypertension in SARD and could support the existence of an overlap between RA and SSc [20].

In summary, anti-hnRNP B1 autoantibodies occur in Russian patients with SARD and particularly in patients with RA and SSc. In the latter patient groups, they seem to be correlated with clinical characteristics such as hypertension. Larger prospective studies are urgently warranted to address the clinical relevance and the pathogenic significance of these autoantibodies.

Abbreviations

ACPA: Anticitrullinated peptide/protein antibody
AI: Augmentation index
BD: Blood donors
CCP: Citrullinated cyclic peptide
CRP: C-reactive protein
DAS28: Disease activity score of 28 joints
ELISA: Enzyme-linked immunosorbent assay
ESR: Erythrocyte sedimentation rate
HYI: Hyperlipidemic individuals
hnRNP: Heterogeneous nuclear ribonucleoprotein
IU: International units
JCR: Juvenile chronic arthritis
PWV: Pulse wave velocity
RA: Rheumatoid arthritis
RF: Rheumatoid factor
RT: Room temperature
RU: Relative units
SARD: Systemic autoimmune rheumatic disease
SLE: Systemic lupus erythematosus
SPA: Spondyloarthropathy
SS: Sjögren's syndrome
SSc: Systemic sclerosis.

Disclosure

Sergey V. Lapin and Dirk Roggenbuck shared senior authorship.

Conflict of Interests

Dirk Roggenbuck is a shareholder of GA Generic Assays GmbH and Medipan GmbH. The remaining authors declare that they have no competing financial interests.

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Review Article

Current Concepts and Future Directions for the Assessment of Autoantibodies to Cellular Antigens Referred to as Anti-Nuclear Antibodies

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The detection of autoantibodies that target intracellular antigens, commonly termed anti-nuclear antibodies (ANA), is a serological hallmark in the diagnosis of systemic autoimmune rheumatic diseases (SARD). Different methods are available for detection of ANA and all bearing their own advantages and limitations. Most laboratories use the indirect immunofluorescence (IIF) assay based on HEp-2 cell substrates. Due to the subjectivity of this diagnostic platform, automated digital reading systems have been developed during the last decade. In addition, solid phase immunoassays using well characterized antigens have gained widespread adoption in high throughput laboratories due to their ease of use and open automation. Despite all the advances in the field of ANA detection and its contribution to the diagnosis of SARD, significant challenges persist. This review provides a comprehensive overview of the current status on ANA testing including automated IIF reading systems and solid phase assays and suggests an approach to interpretation of results and discusses meeting the problems of assay standardization and other persistent challenges.

1. Introduction

In 1950, Coons and Kaplan described the improvement of an immunofluorescence method for the detection of antigens in tissue cells [1]. Eight years later, Friou et al. first described an indirect immunofluorescence (IIF) assay for the detection of anti-nuclear antibodies (ANA) [2, 3]. Along with the earlier discovery of the lupus erythematosus (LE) cell and the development of the LE cell test [4, 5], this ushered in a long and productive age of ANA testing. The ANA IIF test initially relied on rodent tissue substrates but contemporary tests use HEp-2 cells, a cell line established in 1952 by Moore and her colleagues from tumors that had been produced in weanling rats exposed to irradiation and corticosteroids injected with epidermoid carcinoma tissue from the larynx of a 56-year-old male [6]. In the following decades, ANA tests using HEp-2 cells revolutionized the diagnosis of ANA associated rheumatic diseases (AARD) including systemic lupus erythematosus (SLE), systemic sclerosis (SSc), Sjögren's syndrome (SjS), mixed connective tissue disease (MCTD), and idiopathic inflammatory myopathies (IIM) [7, 8].

The IIF assay on HEp-2 cells has been replaced in many laboratories since the development of ANA screening assays based on ELISA and automated, high throughput multiplex assays using addressable laser bead and other array technologies for the detection of specific ANA [9, 10]. Due to a significant prevalence of "false negative" ANA results on these newer platforms and an insufficient communication between laboratorians and clinicians, there have been growing concerns about unilateral adoption of these newer screening and high throughput assays [11]. Questions about which method should be used and the lack of standardization of the novel test algorithms led the American College of Rheumatology (ACR) to form

Table 1: Statistical terms relevant for ANA testing.

Statistical measure	General explanation	Implication for ANA
Sensitivity	Statistical measure of how accurately a test correctly identifies diseased individuals	ANA is used as screening test. High sensitivity is important. The sensitivity for different AARD varies (i.e., higher in systemic lupus erythematosus versus myositis)
Specificity	Statistical measure of how well a test correctly identifies absence of the disease in question	Importance of specificity depends on pretest probability. In settings with low pretest probability, high specificity is required.
Diagnostic efficiency	Combination of sensitivity and specificity	Not commonly used
False negative (clinically)	Negative test result of a diseased individual	ANA is used as screening test. False negative results are undesirable. However, in all AARD, patients without a positive ANA test exist. Therefore, a negative result should never be used to rule out AARD.
False positive (clinically)	Positive test result of an individual without the disease in question	In case of low pretest probability, false positive results significantly impact the posttest probability
False negative (analytically)	Negative test result in the presence of the respective analyte	See negative positive (clinically)
False positive (analytically)	Positive test result in the absence of the respective analyte	See false positive (clinically)
Positive predictive value	Ratio of true positive to combined true and false positives.	Depends on the prevalence (pretest probability)
Negative predictive value	Ratio of true negatives to combined true and false negatives.	Depends on the prevalence (pretest probability)
Positive likelihood ratio	The probability of a positive test results in patients with the disease divided by the probability of a positive test result in individuals without the disease. Independent from prevalence.	[#]Important information for clinicians. Should be included in the laboratory report together with an explanation of its significance in the context of the test result.
Negative likelihood ratio	The probability of a negative test result in patients with the disease divided by the probability of a negative test result in individuals without the disease. Independent from prevalence.	[#]Important information for clinicians. Should be included in the laboratory report together with an explanation of its significance in the context of the test result.

[#]The importance of the likelihood ratio in the laboratory report is controversially discussed, but might improve use of ANA test results in the future.

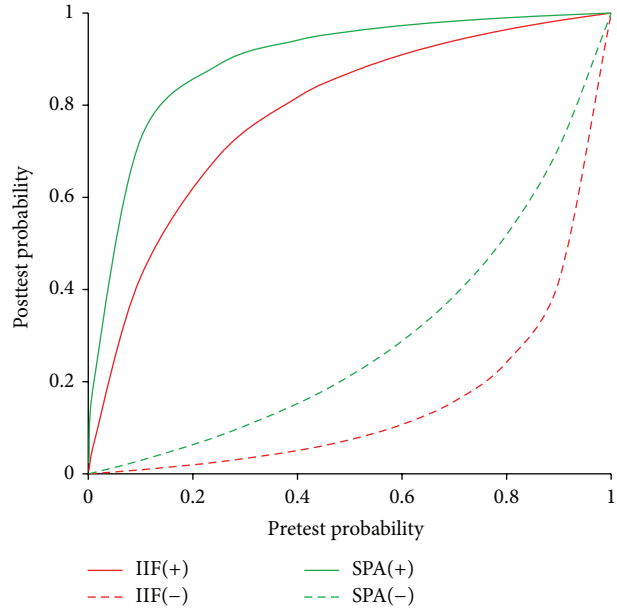
a task force who recommended the use of the conventional IIF HEp-2 platform for ANA detection [12]. This recommendation was, in part, based on evidence that the HEp-2 cell substrates are essentially an "array" presenting >100 autoantibody targets whereas most high throughput screening arrays are much more limited in autoantibody target composition. This has prompted a reevaluation of the ANA IIF method which was reflected by entire sessions dedicated to HEp-2 ANA testing at international clinical and scientific meetings.

In recent years, the first digital imaging systems for ANA IIF have been developed which eliminate some major drawbacks of the method, namely, the subjectivity of observers reading the slides and the lack of an automated procedure [15–17]. Nevertheless, several drawbacks of the HEp-2 IIF methods persist and other technologies for ANA detection continue to emerge and evolve. In this review, novel insights and updates on ANA detection are presented and the pros and cons of different methods are discussed.

2. Statistical Considerations

2.1. Sensitivity and Specificity. For diagnostic applications, it is important to differentiate between analytical sensitivity/specificity and clinical (diagnostic) sensitivity/specificity. Therefore, the terms clinical sensitivity and specificity, false negative, false positive, and predictive values are described in Table 1. In addition, it is widely known and extensively documented that certain autoantibodies can precede the diagnosis or full clinical expression of an underlying disease for many years and thus false positive results at a given point in time might, over a subsequent time period, become a true positive [18, 19]. Consequently, the term "false positive" for autoantibodies needs to be used carefully.

2.2. ROC Analysis and Cut-Off Selection. The receiver operating characteristic (ROC) analysis has a broad range of applications and was first used for military purposes during World War II [20]. In medicine, ROC analysis has been



	Posttest probability			
Pretest probability	IIF(+)	IIF(−)	SPA(+)	SPA(−)
1%: a young woman with hair loss and polyarthralgias	6%	0.08%	19%	0.20%
10%: a young woman with photosensitivity and mild leucopenia (3000–3500 WBC/mm^3)	42%	0.80%	72%	3%
50%: a young woman with photosensitivity, malar rash and symmetrical polyarthritis	87%	7%	96%	21%

Figure 1: Illustration of pretest and posttest probability. Posttest probability (predictive value) for systemic lupus erythematosus as a function of pretest probability and as a function of indirect immunofluorescence (IIF) and solid phase assay (SPA) (EliA CTD screen, Thermo Fisher) test result. Values for likelihood ratios are from Bossuyt and Fieuws [31], WBC = white blood cell.

extensively used for diagnostic testing to evaluate the effectiveness of a novel diagnostic method as compared to an already established one or so called "gold standard" method. Several statistical methods can be applied using the ROC analysis including the most commonly used area under the curve (AUC). The AUC is equivalent to the Mann-Whitney *U*, which tests for the median difference between scores obtained in the two continuous data sets. However, any attempt to summarize the ROC curve into a single number fails as information about the pattern of tradeoffs of the particular discriminator algorithm is not expressed.

The manner by which immunoassay cut-offs are established varies significantly among researchers and scientists in diagnostic companies. A common approach to define the cut-off value for certain assays is to test specimens from patients with the respective disease and compare them to a broad range of controls including related and unrelated diseases as well as age and gender matched (apparently) healthy individuals. The mean value plus 3-fold standard deviation, the 95% or the 99% percentile, of the controls are then often used to define the cut-off value. Another popular approach for definition of the cut-off value makes use of ROC analysis. Despite broad application, most references do not specify how to use the ROC analysis to define the cut-off value [21]. In the majority of cases, a visual approach is used to identify an appropriate point on the ROC curve which provides a good combination of sensitivity and specificity. Following this, it is important that the cohort used to define the cut-off (training set) is large enough to achieve statistical power and that the cut-off is validated using an independent cohort of patients (validation set).

The method used for cut-off definition strongly depends on the assay and how it is intended to be used in a routine setting. For a screening assay, a high degree of sensitivity is mandatory to ensure that the number of patients that are missed by the assay is kept as low as possible. In contrast, confirmation tests need high specificity. In general, low cut-off values increase the sensitivity at the expense of decreasing specificity and *vice versa* when a higher cut-off is defined [9, 10].

The interpretation of the ANA test results depends on the pretest probability of having the disease or whether fulminant disease is present. In a setting of high index of suspicion, even low titers of ANA-IIF can be interpreted as significant [22]. In 1997 it was suggested by Tan and colleagues [23] that the test should be performed and reported at two dilutions, 1 : 40 and 1 : 160, in order to preserve the appropriate sensitivity and specificity. A more recent study recommended using a more

economical single screening dilution of 1 : 160 [24] which was confirmed by a recommendation paper that used a Delphi approach for assessment of autoantibodies to intracellular antigens [21]. ANA-IIF testing on HEp-2 cells is currently widely accepted as the procedure of choice for the detection of ANA but not as a corollary of disease activity or relapse [21].

2.3. Likelihood Ratio. In clinical practice, an important and relevant question is "What is the probability of a patient having a particular disease when the laboratory test is positive or not having the disease when the laboratory test is negative?" [25]. Clinicians and laboratory professionals have difficulties in estimating the posttest probability for a disease based on sensitivity and specificity (2). Likelihood ratio (LR) is an alternative, and probably more easily understood, way to convey diagnostic accuracy data in a clinical setting [26]. The LR for a disease is the probability of the test result in patients with the disease divided by the probability of the same test result in individuals without the disease. The posttest probability for disease associated with a particular test result can be estimated based on the pretest probability and the LR for that particular test result [25, 27].

Traditionally, a single cut-off is used for the interpretation of a laboratory test and all values above or below the cut-off value are given the same interpretation (positive or negative, resp.). For many AARD, the likelihood for disease increases with increasing antibody concentration [28–30]. This information is lacking when a single cut-off is used. LR can be assigned to a particular test result or to a test result interval (e.g., the antibody titer of ANA). It has been shown that the LR for an AARD increases with increasing antibody levels [28]. Knowledge of test result (interval) specific LR improves the clinical interpretation of a particular test result compared to knowledge related to a single cut-off value.

The LR gives an estimation of whether there will be a significant change in pretest to posttest probability of disease given the test result [25]. A LR of 1 implies that there will be no difference between pretest and posttest probability [25]. LRs >10 or <0.1 indicate large, often clinically significant, differences. LRs between 5 and 10 and between 0.1 and 0.2 indicate modest clinical differences [25].

The LR for SLE for ANA by IIF has been estimated to be 7 for a positive test result and 0.03 for a negative test result, whereas the LR for SLE based on solid phase assays (SPA) [in this case: Fluoro enzyme immunoassay (FEIA), EliA CTD screen] has been estimated to be 24 for a positive test result and 0.27 for a negative test result [31]. Using LRs, one can calculate the posttest probability for any given pretest probability [25]. Figure 1 illustrates a graphical representation of the posttest probability (predictive value) for SLE as a function of the pretest probability for IIF as well as for SPA. Such graphical representation has been shown to be a convenient way to convey diagnostic information [26].

To illustrate the impact of pretest probability and assay performance on the posttest probability clinical examples are provided (unpublished data, based on expert experience of Pier-Luigi Meroni).

Table 2: Anti-nuclear antibodies (ANA) in different ANA associated autoimmune rheumatic diseases and healthy individuals.

Antibody	AARD					
	SLE	SSc	SjS	IIM	MCTD	HI
dsDNA	40–70%	<3%	<3%	<3%	<3%	<3%
Chromatin	40–70%	<3%	<3%	<3%	5–18%	<3%
RNP	10–40%	5–15%	<3%	5–15%	100%[5]	<3%
Sm	5–20%	<2%	<1%	<1%	<2%[2]	<1%
SS-A/Ro60	40–70%	3–10%	60–90%	<3%	<3%	<3%
Ro52/ TRIM21	40–70%	15–30%	70–90%	25–50%	<3%	<3%
SS-B/La	15–30%	1–5%	60–80%	5–15%	<3%	<3%
Scl-70 (topo I)	0–5%	20–40%[4]	<3%	<3%	<3%	<1%
Jo-1	1–3%	1–3%	<2%	15–30%	<2%	<1%
Centromere	2–5%	20–40%[4]	5–10%	1–3%	2–5%	<3%
RNA Pol III	<1%	5–25%[4]	<1%	<1%	<1%	<1%
Ribosomal P	10–30%	<2%	<2%	<2%	<2%	<1%
PM/Scl	1–3%	5–10%	<2%	5–10%	<2%	<3%
Mi-2	<1%	3–8%	<1%	5–15%[1]	<1%	<3%
Ku	5–20%	3–8%	<3%	3–10%[3]	<3%	<3%
PCNA	<5%	<1%	<1%	<1%	<1%	<3%
Th/To	<1%	3–10%	<1%	<1%	<1%	<1%

[1]Rare in PM, higher prevalence in DM; mild form of disease; early during development.
[2]Prevalence depends if antigen contains SmBB′ (cross-reactive with RNP).
[3]Very high titer in PM.
[4]Anti-Scl-70, anti-centromere, anti-RNA Pol III antibodies tend to be mutually exclusive.
[5]Part of the classification criteria, therefore should be 100%; however, depending on assay used, some patients might be negative.
Note: Prevalence values were established based on literature and consensus of authors.
Abbreviations: DM: dermatomyositis; IIM: idiopathic inflammatory myopathy (polymyositis/dermatomyositis); MCTD: mixed connective tissue disease; PCNA: proliferating cell nuclear antigen; PM: polymyositis; RA: rheumatoid arthritis; RNA pol III: RNA polymerase III; RNP: ribonucleoprotein; Sm: Smith antigens (U2-U6 RNP); SjS: Sjögren's syndrome; SLE: systemic lupus erythematosus; SPA: Solid phase assay; SSc: systemic sclerosis; TRIM: tripartite motif.

For example, a young woman with hair loss and polyarthralgias, which are very nonspecific signs of SLE, is estimated to have a pretest probability for SLE of 1%. If ANA by IIF turns out to be positive in this patient the probability for SLE increases from 1% to 6%, which is still low. If the SPA CTD screen reveals an autoantibody directed to a specific nuclear antigen (ENA or dsDNA), then the probability for SLE is higher (19%). A second example is a young woman who presents to her physician with photosensitivity and mild leucopenia (3000–3500 WBC/mm^3). Based on this clinical presentation, the probability for SLE is estimated to be 10%. A positive ANA by IIF increases the probability for SLE to 42%, whereas a positive SPA CTD screen increases the probability for SLE to 72%, which makes the diagnosis for SLE likely. However, a woman positive for anti-SSA/Ro could display the same clinical manifestations not necessarily having SLE (sometimes the sicca syndrome is clinically silent at

Table 3: Commercially available immunoassays for ANA testing.

	Automated screening assays				Multiplex assays				Line immunoassays/dot blots					
Antigen	EliA CTD	EliA Symphony	QUANTA Flash ENA7	QUANTA Flash CTD Screen Plus	Alegria ANA Detect	Bio-Plex 2200 ANA ALBIA	AtheNA Multi-Lyte Anti-Nuclear Antibodies (ANA)	FIDIS Connective 10	ANA Profile	ImmcoStripe ANA Advanced	ANA12 IgG BlueDot	ANA-12 Pro (BLOT)	recomLine ANA/ENA IgG	ANA Lia MAX
	Thermo Scientific	Thermo Scientific	INOVA Diagnostics	INOVA Diagnostics	Orgentec	Bio-Rad	Zeus	Theradiag	Euroimmun	IMMCO Diagnostics	D-Tek	AESKU Diagnostics	MIKROGEN Diagnostics	HUMAN Diagnostics
U1-RNP	X	X	X	X		X	X		X		X	X	X	
U1-RNP/Sm		X	X	X	X			X			X			
U1-RNP 68 kDa					X					X				X
U1-RNP A					X					X				X
U1-RNP C					X					X				X
SS-A/Ro60	X	X	X	X	X	X[(i)]	X	X	X	X	X	X	X	X
Ro52/TRIM21	X	X	X	X	X	X[(i)]			X	X		X	X	X
SS-B/La	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Centromere	X	X		X[#]	X	X	X	X		X	X[#]	X	X	X
Scl-70/topo I	X	X	X	X	X	X	X	X	X		X	X	X	X
Jo-1	X	X	X	X	X	X	X	X	X	X	X	X	X	X
Fibrillarin/U3RNP	X													
RNA-Pol III	X			X										
Ribosomal P	X			X		X		X	X	X	X	X	X	X
PM/Scl	X			X					X					X
PM/Scl-75										X				
PM/Scl-100					X					X				
PCNA	X			X					X	X	X		X	X
Mi-2	X			X					X	X				X
Sm	X			X	X	X	X		X	X	X	X	X	X
dsDNA	X			X	X	X		X	X	X		X	X	X
Nucleosome					X				X	X		X		X
Histone					X		X		X	X		X	X	X
Ku				X					X	X	X			X
SRP54										X				
AMA-M2									X	X				X

Note: some companies offer several line immunoassays for ANA detection. Most comprehensive assays from different companies are shown.
AMA: Anti-mitochondrial antibodies; PCNA: Proliferating cell nuclear antigen; SRP: signal recognition particle.
NOTE: RNP and RNP/Sm contain the subunits RNP-A, RNP-C and RNP-68 kDa.
[(i)]Reported outside the United States as Ro60 and Ro52 and as SS-A in the United States.
[#]Contains CENP-A and CENP-B.

the beginning of a primary SjS). A negative ANA by IIF result would reduce the probability for SLE from 10% to <1%. The third example is a young woman with photosensitivity, malar rash, and symmetrical polyarthritis. This clinical picture is suggestive of SLE (50% probability). A positive IIF result increases the probability for SLE from 50% to 87%, whereas a positive SPA CTD screen increases it to 96%. A negative IIF result would reduce the probability for SLE to 7%, whereas a negative SPA test result would reduce the probability of SLE to 21%, illustrating the NPV of SPA is lower than the NPV of IIF.

3. Nomenclature of Antibodies to Cellular Antigens Commonly Referred to as Anti-Nuclear Antibodies (ANA)

Historically, only antibodies targeting antigens present in the nuclear compartment of the cells (nuclear antigens) were called ANA. Similarly, the term extractable nuclear antigen (ENA), described in 1959 by Holman and Robbins was used for a group of nuclear antigens extractable by saline solutions [14]. Nowadays, with the identification of a variety of new autoantigens within various compartments of the cell, the nomenclature has become rather imprecise and misleading [21]. As an oversimplification, even serum samples with anti-cytoplasmic but without ANA reactivity are sometimes considered as ANA positive [32–34]. This confusing terminology has even been adopted in the nomenclature of commercial autoantibody assays and kits. Autoantibody arrays on various technology platforms are often termed as ANA or ENA profiles even though they contain relatively insoluble nuclear antigens such as dsDNA and/or cytoplasmic targets such as ribosomal P or Jo-1 antigens. Therefore, standardization of this nomenclature is highly desirable.

4. Anti-Nuclear Antibodies in Different Conditions

To date, more than 160 autoantigens, many of them localized to the cell nucleus, have been described in sera of SLE patients [35]. Therefore, the spectrum of SLE associated autoantigens contained in most ANA screening SPA includes only a small proportion of antigens targeted by SLE autoantibodies [35, 36]. However, most SLE associated autoantigens, apart from the standard ENAs, are rarely the target of individual SLE sera and even more uncommon without reactivity to any of the standard ENAs. For example, a recent study found a sensitivity of a SPA for SLE of 79% (in diagnostic samples) compared to a sensitivity of IIF at cut-off 1 : 160 of 90% [29]. Thus, the number of SLE patients having at least one clinically meaningful autoantibody that are missed by ANA SPA appears to be approximately 10%. In contrast, an even larger proportion of SSc patients have a negative test results when an ANA SPA is used [37]. Consequently, the clinical utility of novel assays for different AARD can be different and each new assay has to be validated in all AARD subgroups (SLE, SSc, MCTD, SjS, and IIM).

In addition to SLE and SSc, ANA can be found in various other SARD including but not limited to IIM, SjS, and MCTD (Table 2). The appreciation that ANA are useful diagnostic biomarkers in a broad spectrum of autoimmune conditions has led to a significant change in the referral pattern of ANA tests to diagnostic laboratories (see Figure 1). Historically, primarily rheumatologists and clinical immunologists ordered ANA testing as an aid to the diagnosis of SLE. Much of this was due to the embedding of ANA and certain ENA in the older and now more recent classification criteria for SLE [38, 39]. Nowadays, a wider spectrum of clinicians order the ANA test (see Figure 1) including but not limited to internists, dermatologists, nephrologists, oncologists, cardiologists, neurologists, gastroenterologists, otolaryngologists, ophthalmologists, gynecologists, and even primary care physicians (Figure 2). This can be attributed to the broadening spectrum of ANAs in rheumatoid arthritis (RA), antiphospholipid syndrome (APS) [40], autoimmune liver diseases such as autoimmune hepatitis and primary biliary cirrhosis (PBC) [41–46], vasculitis [47], inflammatory bowel disease (IBD) [48–51], and cancer [52–57] (Table 4).

5. Differential Diagnosis of Autoimmune Diseases

The early and accurate diagnosis of autoimmune diseases can be very challenging because the spectrum of signs and symptoms are very wide and often overlap. Initially, an AARD has to be differentiated from a wide spectrum disorders (i.e., infections, malignancies, allergic, and adverse drug reactions) presenting with similar signs and symptoms. For example, a patient suspected to have SLE can first present with skin manifestations which need to be differentiated from discoid lupus, polymorphous light eruption, rosacea, drug eruptions, and other dermatoses. If other organs are involved (i.e., kidney, lung, musculoskeletal, cardiovascular, or neuropsychiatric [58]) the differential diagnosis must take other diagnostic possibilities into consideration. Secondly, after the presence of an AARD is confirmed on the basis of signs, symptoms, and physical examination, the different AARD need to be differentiated from each other so as to assist the clinician with decisions about appropriate therapeutic interventions. This can be further complicated by the evolution of autoimmune diseases from one condition to another. Many AARD, especially SLE, can present with arthritis but during follow-up, a diagnosis of RA or "rupus" might be established [59, 60]. Similarly, MCTD can evolve into SSc or RA. The appropriate interpretation of a positive or negative ANA can help enlighten the diagnostic and prognostic accuracy of AARD, although very little is known about the LR to differentiate the different diseases.

6. Screening and Profile Assays for ANA Detection

6.1. ANA by Indirect Immunofluorescence on HEp-2 Cells. For well over the last decade, the IIF HEp-2 assay was being replaced by newer technologies for the detection of ANA [61]

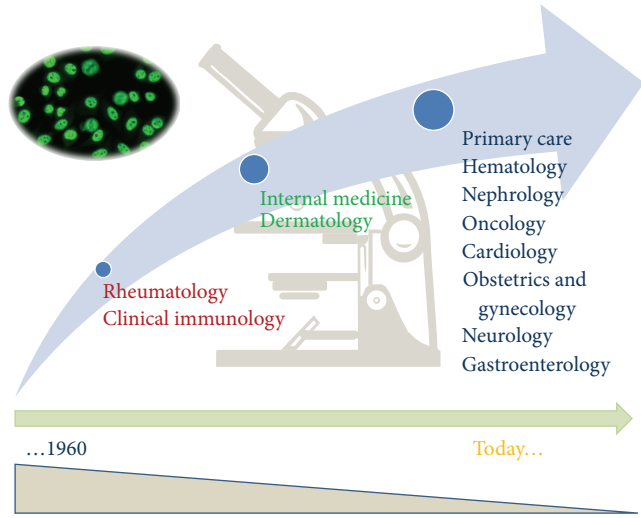


Figure 2: Change in referral patterns. Historically, when the ANA HEp-2 test became available in around 1960 exclusively rheumatologist and clinical immunologists ordered the ANA test. With the emerging recognition that many other diseases are associated with ANAs, a broad range of clinical disciplines order the ANA test. With changes in the ANA referral pattern and the associated decrease in the pretest probability, the posttest probability significantly decreases (indicated by the triangle).

Table 4: Clinical utility of ANA testing in different diseases.

Diagnosis	Clinical utility	ANA prevalence	Monitoring/prognosis	Comments
SLE	Very useful	90–95%	Not useful	ANA IIF superior to ANA solid phase assays
SSc	Very useful	85–95%	Not useful	ANA IIF superior to ANA solid phase assays
SjS	Useful	50–60%	Not useful	ANA solid phase assays superior to ANA IIF; SS-A reactivity can be missed by ANA HEp-2
AIM	Somewhat useful	50–60%	Not useful	ANA solid phase assays superior to ANA IIF; Jo-1 reactivity can be missed by ANA HEp-2
MCTD	Very useful	90–100%	Not useful	High titer anti-U1-RNP are highly indicative for MCTD
JCA/JIA	Somewhat useful	50–60%	Very useful	Useful for subset that are at risk of developing uveitis
PBC	Very useful	50–80%	Not proven	ANA IIF superior to solid phase assays; Antibodies to SP100, gp210, nucleoporin p62, lamin B receptor and Ro52 /TRIM21. Anti-gp210 reported association with poor prognosis.
RA	Not useful	15–20%	Not useful	Homogeneous and speckled staining are the most common patterns
APS	Not useful	40–70%	Not useful	Might indicate systemic autoimmunity in primary APS patients
AT	Not useful	10–20%	Not useful	Higher in Grave's disease as compared to Hashimoto's thyroiditis
Cancer and paraneoplastic syndromes	Not useful, or utility not established	20–50%	Not useful	Antibodies to CENP-F and to other proteins might be useful to help in the diagnosis of cancer; p53 has been discussed; not many systematic studies on ANA in cancer
AIH	Useful	40–80%	Not useful	Prevalence depends on phase of the disease

Abbreviations: AIH: autoimmune hepatitis; AIM: autoimmune inflammatory myopathy (polymyositis, dermatomyositis); APS: anti-phospholipid syndrome; AT: autoimmune thyroiditis; JCA/JIA: juvenile chronic arthritis/juvenile inflammatory arthritis; MCTD: mixed connective tissue disease; PBC: primary biliary cirrhosis; RA: rheumatoid arthritis; SjS: Sjögren's syndrome; SLE: systemic lupus erythematosus; SSc: systemic sclerosis NOTE: Prevalence values are based on diagnostic samples (not treated patients).

Table 5: Advantages and disadvantages of the HEp-2 ANA test.

Advantages	Disadvantages
Variety of different target autoantigens (>100)	Subjectivity
Some autoantibodies can be identified without confirmatory testing (i.e., anti-centromere)	Poorly standardized across manufacturers
Discovery tool for novel autoantibodies	Requires training and expertise
Useful for a spectrum autoimmune diseases (i.e., autoimmune hepatitis)	Low sensitivity for certain clinically important autoantibodies (i.e., Jo-1, ribosomal P, SS-A/Ro60, Ro52/TRIM21)
	Low specificity (high false positive rate)

Table 6: Overview of defined ANA patterns (modified from Wiik et al., 2010 [13]).

Pattern group	Pattern
Nuclear envelope (membrane)	Smooth nuclear envelope
	Punctate nuclear envelope
Nuclear	Homogeneous pattern
	Large speckled
	Coarse speckled
	Fine speckled
	Fine grainy Scl-70-like
	Pleomorphic speckled (i.e., PCNA)
	Centromere
	Multiple nuclear dots
	Coiled bodies (few nuclear dots)
	Dense fine speckled
	Isolated metaphase chromosomes
Nucleolar	Homogeneous nucleolar
	Clumpy nucleolar
	Punctate nucleolar
Mitotic spindle apparatus	Centriole (centrosome)
	Spindle pole (NuMa) (MSA-1) (HSeg5)
	Spindle fiber
	Midbody (MSA-2)
	CENP-F (MSA-3)
Cytoplasmic	Diffuse
	Fine speckled
	Mitochondrial
	Discrete dots: GW bodies, endosomes, lysosomes
	Golgi complex
	Intercellular contact proteins
	Fibers and cytoskeleton
	Rods and rings
Negative	

and several larger laboratories switched to automated high-throughput immunoassay platforms [61]. However, in 2010, a position paper was published indicating that IIF on HEp-2 cells should remain the "gold standard" for the detection of ANA [12], triggering a renaissance of the IIF ANA test. Nevertheless, in some cases, an ANA result based on IIF ANA on HEp-2 substrates may mislead the clinician and has to be interpreted within the clinical context [62] (Table 5). In addition, standardization of this assay is difficult due to intermanufacturer variations in the substrate and the fixation process, characteristics of the secondary antibody used [63], interlaboratory variations in microscopy apparatus, and, especially, the subjective interpretation of the results [64]. Detection of ANA by IIF may also yield false negative results even in the presence of high titers of antibodies, such as those directed to SS-A/Ro60, Ro52/TRIM21, Jo-1 (histidyl tRNA synthetase), and others [65–67]. Additionally, the challenge of significant variation of staining patterns on the ANA HEp-2 IIF substrates obtained with slides from different manufacturers [63] has led to a proposed nomenclature for IIF patterns [13] (Table 6). For these reasons, considerable effort has been dedicated to the development of standardized SPA for routine use, such as ELISA [68], which are attended by guidelines for the detection of ANA [21, 62, 69].

6.1.1. Automated Pattern Recognition of the ANA HEp-2 Test. Computer assisted pattern recognition for ANA testing on HEp-2 cells has been described more than ten years ago [70]. Automated hardware and software-based pattern recognition platforms that allow for the identification and archiving of IIF patterns obtained on HEp-2 cell substrates; however, they have only become available during the last few years [15, 16, 71–74]. The operating principle of these new automated systems is acquiring, storing, and analyzing of digital images of stained IIF slides and displaying them on high resolution computer monitors. The inherent technical difficulties of processing and reading IIF slides (manual reading, real-time interpretation, need for dark room, and handwritten results transcription) make traditional IIF methods difficult to fit in the workflow of modern, automated laboratories. The new automated systems are powerful workflow and operational tools that can eliminate the need for a darkroom and separate image acquisition from image interpretation and have the potential to improve the quality and utility of the ANA HEp-2 assay.

The currently available automated ANA IIF image analyzing systems include NOVA View (INOVA Diagnostics, San Diego, US) [30], Aklides (Medipan, Berlin, Germany) [15, 16, 75], G-Sight (Menarini, Florence, Italy) [76, 77],

Table 7: Automated digital ANA reading systems.

Instrument	NOVA View	AKLIDES	EUROPattern	Image Navigator	Helios	ZENIT G Sight
Manufacturer	INOVA diagnostics	Medipan	Euroimmun	Immunoconcepts	Aesku	Menarini
LIMS connection (software)	Yes (QUANTA Link)	Yes (system independent, standard XML interface)	Yes (EUROLabOffice)	Yes (direct) Optional: lab traffic control	Yes (direct) Optional: Aesku.Lab middleware	Yes (ZenIT)
Slide identification via barcode	Yes by handheld scanner	Yes by handheld scanner	Yes by integrated scanner	Yes	Yes by integrated scanner	Yes by integrated scanner
Loading capacity	5 slides (up to 60 wells)	5 slides (up to 60 wells)	50 slides (up to 500 wells)	4 slides (up to 84 wells)	20 slides (up to 240 wells)	5 slides (up to 70 wells)
Image acquisition speed	~45 s/well for 3 images	~40 s/well	<20 s/well	~25 s/well for 4 images	10 s/picture Customizable from 1 to 10 images	>60 s/well number of pictures: 5 (small scan), 50 (medium scan), or 220 (full scan)
100% QC for substrate and process integrity/counterstaining	Yes/DAPI	Yes/DAPI	Yes/Propidium iodide	None/None	None/None	None/None
Automatic pos./neg. discrimination incl. presorting of images	Yes	Yes	Yes	Yes	Yes	Yes
Batchwise verification of negative samples	Yes	Yes	Yes	Yes	Yes	Yes
Automatic pattern recognition	Yes	Yes	Yes	No	No	Yes
Pattern Analysis method	Pattern recognition by mathematical algorithm	Pattern recognition by mathematical algorithm	Pattern recognition by mathematical algorithm	No pattern matching capabilities	No pattern matching capabilities	Pattern recognition by mathematical algorithm
Number of recognizable ANA staining pattern list out	6 Homogeneous Speckled Centromere Nucleolar Nuclear dot Cytoplasm Negative Positive unrecognized	10 Homogeneous Speckled Centromere Nucleolar Nuclear dot Cytoplasm Negative Positive unrecognized	8 Homogeneous Speckled Centromere Nucleolar Nuclear dot Cytoplasm Negative Nuclear rim Mixed pattern mitotic	None	None	5 Homogeneous Speckled Centromere Nucleolar Cytoplasm Negative
Analysis of mixed staining pattern	Limited (homogeneous/ nucleolar)	Yes	Yes	No	No	No
Merged results per patient (different dilutions)	Yes	Yes	Yes	Yes	Yes	Yes
Final result validation possible while system processes remaining samples	Yes	No	Yes	Yes	Yes	No
Instrument calibration to minimize variability	Yes	Yes	Yes	Yes	Yes	No
Integration with slide processing in 1 instrument	No	No	No	No	Yes	No

EuroPattern (Euroimmun, Lübeck, Germany) [73], Image Navigator (ImmunoConcepts, Sacramento, US), and Helios (Aesku, Wendelsheim, Germany) (Table 7). The systems differ from each other with respect to the use of DNA-binding counterstains, such as DAPI, the cell substrate used (e.g., most systems are restricted to using the respective manufacturer's slides), the throughput, the number of patterns that can be identified, and user-friendly features of the software [76, 77].

Generally, these automated systems are based on a microscope fitted with an automated stage, a CCD digital camera, a LED light source, and software that controls the moving parts and directs image acquisition. All systems perform some kind of fluorescent light intensity measurement and use the results for preliminarily categorization of the samples as positive or negative and for pattern analysis. The automated reading is followed by human visual interpretation of the digital images that are displayed on a computer monitor, allowing user confirmation or revision of the automated results. By providing good quality digital images and other objective information (such as preliminary classification and pattern interpretation), these automated systems support the operators' decision making and increase the consistency between readers and readings. In addition, the digital images can be stored for training, documentation, follow-up, and second opinion purposes. In the future, these digital images might also become part of the patient's electronic medical record (EMR). At present, the systems are highly reliable in their ability to discriminate positive from negative reactions and to estimate fluorescence intensity, but the accuracy and robustness of pattern recognition does not reach the accuracy of human interpretation [77, 78]. An important feature is, the quality of the digital images, enabling the operators to make the same clinically relevant interpretation as they would make using a conventional microscope.

The final result interpretation is made by the operator, therefore subjectivity cannot be completely removed. Moreover, the characteristics of the HEp-2 substrates and conjugates influence the appearance of certain ANA specificities, and the automated pattern identification of the various systems is likely based on somewhat different programming principals. Nevertheless, automated systems for ANA HEp-2 analysis are a significant step forward to reduce variability and offer opportunities to increase harmonization of ANA interpretation [79].

Some systems offer automated assessment of ANA endpoint titers on a single serum dilution, thereby eliminating the need for serial dilutions [75]. Moreover, the potential integration of the automated digital IIF systems with laboratory information systems (LIS) provides sample traceability, and eliminates manual transcription and associated transmittal errors, thereby improving patient safety. The systems also hold the promise to reduce hands on time. Work flow studies using different systems are required to analyze the efficiency benefits of those systems.

6.1.2. Limited Sensitivity and Specificity of ANA HEp-2 IIF Test. Several studies have demonstrated limited analytical/clinical sensitivity [61, 63, 66, 80, 81] and clinical specificity [82–85], of IIF on HEp-2 cells. In particular for anti-Rib-P, anti-SSA/Ro60, and anti-Jo-1 autoantibodies, the ANA HEp-2 IIF test has been reported to lack analytical sensitivity which translates to the clinical sensitivity for AARD [63, 65, 66, 81, 86, 87]. In a comparative analysis of an ANA ELISA and ANA IIF, equivalent sensitivity but significantly higher specificity of the ELISA was observed [84]. When using the cut-off recommended by the manufacturer, the clinical specificity of IIF for AARD was as low as 62.3% [84].

Based on these observations, over three decades ago a transfected HEp-2 cell line overexpressing SS-A/Ro60 was developed and this cell-based IIF assay is marketed as HEp-2000 cells [81, 88–90]. A similar approach has recently been used for SmD1 antigen in a research HEp-2 cell line [91]. This technological approach of overexpressing target antigens in a variety of cell lines has more recently become a productive approach to cell based IIF assays where structural or conformational epitopes are important for human autoantibody detection. It was found that adding antigen specific assays to IIF on HEp-2 cells significantly improved the diagnostic algorithm for the diagnosis of SARD [92]. However, it was concluded in this study that changing from IIF to other methods for ANA detection also required modification of the disease criteria. In addition, it was highly recommended to use anti-SS-A antibody assays in addition to ANA HEp-2 test [92].

6.2. Different Staining Patterns Have Different Specificities. Disease classification criteria, such as the SLE criteria [38, 39], do not distinguish between different ANA IIF patterns. Although certain ANA patterns on HEp-2 cells have a significant disease associations, in clinical practice differentiation of IIF patterns is rarely used as an aid in establishing the clinical diagnosis [21]. Nevertheless, it is well established that the centromere staining pattern is primarily associated with the limited cutaneous form of SSc (also referred to as the CREST syndrome) [8, 93]. Additional examples are the association of the homogenous IIF pattern with SLE and nucleolar IIF pattern with SSc [37], although these generalizations have not been observed in all studies [94] (most likely attributed to different pretest probabilities). Interestingly, antibodies to dense fine speckled 70 (DFS70), also known as LEDGF (lens epithelium-derived growth factor), which generate a DFS IIF pattern on HEp-2 cells were not commonly observed in AARDs [82, 95–102]. These antibodies decorate interphase nucleoplasm outside of the nucleolus but in contrast to the anti-SSA/SSB and anti-Mi-2 antibodies, the anti-DFS70/LEDGF antibodies stain the metaphase and telophase chromosomal cell plates [13] (Figure 3). Just recently, it was confirmed that the DFS pattern was not associated with AARD and was primarily found in apparently healthy individuals [103]. In rare cases, this pattern can also be observed in patients with AARD, but these cases, anti-DFS70 antibodies are commonly accompanied by other autoantibodies [98, 102]. Follow-up of individuals with high titers of anti-DFS70 antibodies revealed that they retain ANA reactivity even after four years but do not develop SARD [103, 104]. Hence, it has been suggested that, within some

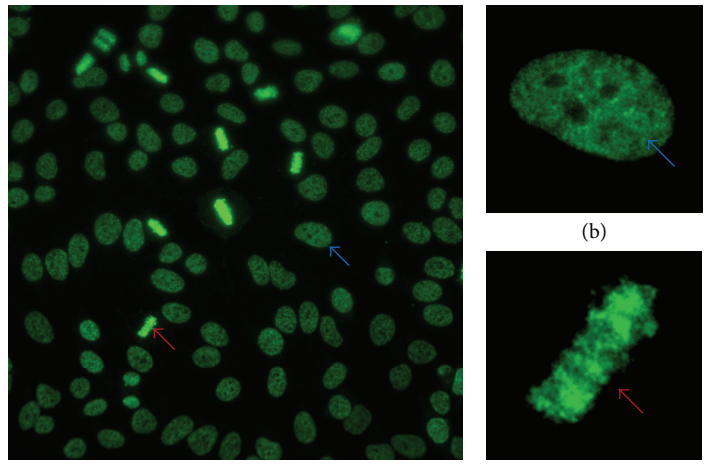


Figure 3: Characteristic staining pattern of anti-DFS70 antibodies. The characteristic dense fine speckled (DFS) staining pattern of interphase HEp-2 cells is indicated by the blue arrow and the strong chromatin staining of mitotic cells by the red arrow. (a) Wide field view using 40x magnification, (b) dense fine speckled pattern of an interphase nucleus, and (c) of the metaphase chromatin of a mitotic cell.

limits, anti-DFS70 antibodies can be used to exclude the diagnosis of AARD [82]. The major epitope of the molecule is conformation dependent and is located in the C-terminal part of the molecule [105]. Although the immunoreactive region has been shown to be located within a stretch of 22 amino acids (407–435), the use of 12 mer peptides failed to establish reactivity with the presumed linear epitope [105].

6.3. ANA Screening ELISA. During the last decade, different strategies have been utilized to develop, evaluate, and commercialize several ANA screening ELISAs [106–110]. The majority of ANA screening ELISAs make use of mixtures or "blends" of purified autoantigens from native sources and/or recombinant technologies [106, 111]. The composition of these antigen preparations is quite diverse and is dependent on several factors including the availability of pure antigens and the technical feasibility of combining all different antigens in a single assay. Most available immunoassays contain SS-A/Ro60 [112], SS-B/La, Scl-70/topoisomerase I [113], CENP-B [93], Jo-1, U1-RNP, Sm, and dsDNA [114]. ANA screening ELISAs from some manufacturers also contain other autoantigens such as PM/Scl or ribosomal P [36]. However, based on comparison to immunoprecipitation of radiolabelled native proteins and other techniques, reactivity can be missed by these ELISAs even if the autoantigens are contained in the mixture [36]. This can be attributed to the notion that individual autoantigens exhibit different biochemical properties and, therefore, display different binding behaviors when solid phase matrices are used to bind the target autoantigens. For example, some antigens might bind to other targets in the same mixture resulting in a masking effect that gives the impression that insufficient epitopes are available for human autoantibody binding. Some antigens, such as PCNA [115, 116], RNA Pol III [117], or Th/To [118], are rarely included as purified antigen in screening assays.

6.4. Line Immunoassays. Line immunoassays (LIA) can basically be considered second generation dot-blot assays. A broad range of LIAs are available and they are typically used to confirm autoantibodies previously identified by HEp-2 ANA IIF or other screening immunoassays [119]. However, in some laboratories, these LIAs have also been used as a screening test for disease specific autoantibodies that are seen in SLE, SSc, IIM, paraneoplastic, and autoimmune liver diseases [120]. There have been recent advances in partially automating these multiplex LIAs making them somewhat more appealing to high throughput laboratory testing [121]. Despite their ease of use, LIAs have some drawbacks including the lack of sensitivity and specificity for certain autoantibodies [120, 122]. The antigen compositions of several are shown in Table 3.

6.5. Multiplex Bead-Based Assays. Multiplex assays based on the Luminex technology (Austin, Texas, USA) use addressable laser beads and are therefore often referred to as ALBIA (addressable laser bead immunoassays) [9]. Today, several commercial ALBIA kits are available for the detection of autoantibodies to a variety of autoantigens [83, 85, 123–130]. First generation ALBIAs showed polyreactivity which was caused by nonspecific binding to the beads [131]. Second generation assays showed significant reduced polyreactivity and thus higher specificity [131]. In 2007, a multiplex test for the detection of ANA was compared to ANA IIF and different ELISA assays. 7/87 (7.4%) of healthy donors were positive, 6/7 showed a speckled, and 1/7 a nucleolar staining pattern [85]. Similar to LIAs, the number of antigens and the antigen compositions of these bead-based arrays significantly vary and are shown in Table 3.

6.6. Other ANA Tests. Additional methods have been developed for automated ANA detection [84, 108, 132–139]. In

1999, a fully automated ANA screening assay (COBAS Core HEp2 ANA EIA; Roche Diagnostics, Mannheim, Germany) was developed and evaluated. The performance evaluation studies showed promising results but were inclusive in the conclusion. One study shows that the new assay is superior to the ANA IIF as analyzed by ROC analysis [138]. However, this finding could not be confirmed in a second independent analysis [133]. Six years later, the first-automated chemiluminescent immunoassay (CIA) for the detection of ANA (LIAISON ANA screen, DiaSorin) was evaluated in two centers yielding a good positive (79.5%) and negative agreement (91.2%) when the LIAISON ANA screen was compared to the ANA IIF test (Bio-Rad) [140]. A recent study using this assay showed ANA prevalence compatible with the expected values of the ANA IIF test [42]. Only moderate agreement was found between IIF and a multiplex assay based on the ALBIA. The majority of ANA IIF positive and multiplex ANA negative sera were also positive by an ANA ELISA utilizing nuclear extracts [129]. In addition, several other methods have been developed for ANA testing, but are not widely used in clinical practice. In 2009, a novel method for quantitative ANA measurement using near-infrared imaging was also described [141]. Furthermore, a novel microbead-based ELISA system using fluorescence-coded immobilized microbeads on the AKLIDIS system has been described [142]. Similar to the ALBIA, but using nanobarcodes for the bead identification, the Ultraplex system [143] was used to screen simultaneously for nine ANA autoantibodies, requiring significantly less labor and fewer reagents, with performance equivalent to existing gold-standard methods.

Several publications have described protein arrays on planar solid phase surfaces for the detection of autoantibodies to a wide range of viral proteins and autoantigens [144, 145] bound to a variety of surfaces [146]. However, these immunoassays are still not used in routine diagnostic laboratories.

More recently novel "ANA" screening assays have been developed on fully automated closed systems such as the Phadia (Thermo Fisher, Freiburg, Germany) or the BIO-FLASH System (INOVA, San Diego, USA) [147]. The EliA CTD Screen (Thermo Fisher) has been evaluated in several studies, two of which have been published in peer-reviewed journals [28, 148]. The first study showed satisfactory results for anti-ribosomal P, anti-PM/Scl, anti-Mi-2, and anti-PCNA antibodies. However, the sensitivity for anti-fibrillarin and anti-RNA Pol III antibodies was rather limited [148]. In the second study, the CTD screen was compared to the ANA HEp-2000 method [29]. Additionally, the QUANTA Flash CTD Screen Plus (on BIO-FLASH) was evaluated and shown to exhibit good sensitivity in different SARD [149]. However, further studies are needed to establish the clinical performance characteristics of the novel assays.

7. Quality Aspects, Standardization, and Reference Sera

For the development and quality assurance of autoantibody assays a broad range of international reference samples are mandatory [150, 151]. Consequently, an ANA and related autoantibody reference serum panel was established and is now available through the Center for Disease Control and Prevention in Atlanta, USA [152–155]. Initially, this panel of sera was used to standardize ANA IIF tests and to define staining patterns, but eventually, this panel was used to evaluate the performance of different autoantibody immunoassays [152]. In 2000, another ANA reference serum panel became available through the Association of Medical Laboratory Immunologists (AMLI) [156]. However, only a few studies used these samples for their investigations [157]. The first international standard for ANA became available in 1990 [158]. However, the number of international reference sera is limited and not all autoantibodies are represented by the available serum panels [150]. Furthermore, for screening assays, monospecific samples for each antigen are required to ensure the presence of all antigens in sufficient quantity and quality.

Different committees and organizations were formed who worked to achieve a better standardized approach to autoantibody testing [150, 159, 160] including the International Union of Immunology Specialties (IUIS) Autoantibody Standardization Committee (http://asc.dental.ufl.edu/home.html#text), the European Autoimmunity Stand-ardisation Initiative (EASI) [http://www.easi-network.com/], and the Working Group on Harmonization of Autoantibody Tests (WG-HAT) in the framework of the International Federation of Clinical Chemistry and Laboratory Medicine [http://www.ifcc.org/ifcc-scientific-division/sd-working-groups/harmo-onisation-of-autoantibody-tests-wg-hat/]. Despite significant efforts to standardize autoantibody tests [152, 161] and evidence that these groups are now working more closely together, [21] significant variations still exist.

8. Conclusions

(i) Performance data (including LRs) of the method used to detect ANA and appropriate explanation should be made available to the clinician.

(ii) ANA test results are only a portion of the information that aids in the diagnosis of systemic autoimmune diseases and are an adjunct to the clinician's diagnostic repertoire.

(iii) Both IIF on HEp-2 cells and solid phase immunoassays have their individual advantages and limitations.

(iv) Standardized nomenclature of diseases and associated autoantibodies is an important goal for immediate consideration by advisory groups.

9. Future Perspectives

Despite significant evolution and improvements in ANA and related autoantibody testing, including the arrival of novel and promising technologies, several limitations still persist and need to be addressed. First, the terminology and nomenclature used to identify and refer to various autoantibodies need to be standardized. Second, the classification criteria and nomenclature of individual SARD

and related autoimmune diseases must continue to evolve and keep abreast of biomarker identification. Third, the corresponding immunoassays and diagnostic platforms used for the various clinical applications need to be based on standardized reference samples of defined specificities. This possibility could include the development and validation of disease specific screening assays (i.e., SLE Screen, SSc Screen) on solid phase technologies. Fourth, a clearly defined strategy needs to be developed to facilitate clinicians and laboratory scientists alike becoming more familiar with and be able to intelligently use objective interpretation of autoantibody results through an understanding of ROCs and LRs. Lastly, diagnostic algorithms need to be adjusted to the clinical and laboratory setting considering the referral pattern, the sample testing volume, and health economic aspects (i.e., reimbursement).

Conflict of Interests

Michael Mahler is employed at INOVA Diagnostics, a company that manufactures and markets autoantibody assays. Pier-Luigi Meroni received fees as consultant for INOVA Diagnostics and from BioRad. Xavier Bossuyt has been a consultant to INOVA Diagnostics and has received lecture fees from Instrumentation Laboratory, Thermo Fisher, and Menarini. Marvin J. Fritzler is the Director of Mitogen Advanced Diagnostics Laboratory (Calgary, Alberta, Canada) which performs autoantibody testing and is also a consultant to INOVA Diagnostics, and has received gifts in kind from Euroimmun GmbH.

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Research Article

Chinese SLE Treatment and Research Group Registry: III. Association of Autoantibodies with Clinical Manifestations in Chinese Patients with Systemic Lupus Erythematosus

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We investigated the characteristics of Chinese SLE patients by analyzing the association between specific autoantibodies and clinical manifestations of 2104 SLE patients from registry data of CSTAR cohort. Significant ($P < 0.05$) associations were found between anti-Sm antibody, anti-rRNP antibody, and malar rash; between anti-RNP antibody, anti-SSA antibody, and pulmonary arterial hypertension (PAH); between anti-SSB antibody and hematologic involvement; and between anti-dsDNA antibody and nephropathy. APL antibody was associated with hematologic involvement, interstitial lung disease, and a lower prevalence of oral ulcerations ($P < 0.05$). Associations were also found between anti-dsDNA antibody and a lower prevalence of photosensitivity, and between anti-SSA antibody and a lower prevalence of nephropathy ($P < 0.05$). Most of these findings were consistent with other studies in the literature but this study is the first report on the association between anti-SSA and a lower prevalence of nephropathy. The correlations of specific autoantibodies and clinical manifestations could provide clues for physicians to predict organ damages in SLE patients. We suggest that a thorough screening of autoantibodies should be carried out when the diagnosis of SLE is established, and repeated echocardiography annually in SLE patients with anti-RNP or anti-SSA antibody should be performed.

1. Introduction

Systemic lupus erythematosus (SLE) is one of the most complicated autoimmune diseases. It could involve almost all organs or systems and presents with protean clinical manifestations [1]. In general, SLE can be divided into several subgroups based on specific clinical features including age, gender, and autoantibodies pattern, and the prognosis of different subgroups varies [2]. Anti-Sm antibody is considered as the marker autoantibody for the diagnosis of SLE with reported positivity ranged from 15.4% to 21.8% [3, 4]. Anti-double stranded DNA (anti-dsDNA) antibody is another specific autoantibody for SLE and has been proven to be associated with disease activity of SLE [5]. In order to understand SLE better, the association between clinical features of SLE and other anti-extractable nuclear antigen (ENA) antibodies (e.g., anti-SSA, anti-SSB, anti-RNP, and anti-rRNP antibodies) had been investigated by many research groups [6, 7]. In our study, we analyzed the associations between clinical manifestations and autoantibody patterns in Chinese SLE patients based on the data from Chinese SLE Treatment and Research group (CSTAR) registry. CSTAR is the first online registry of Chinese SLE patients and is supported by the Chinese National Key Technology R&D Program. This registry has depicted major clinical characteristics of lupus in Chinese patients [8].

2. Methods and Patients

2.1. Patients. CSTAR launched the first registry project of Chinese SLE patients in 2009, which was approved by the Institute Review Board (IRB) of Peking Union Medical College Hospital (PUMCH). Other centers had received ethical approval by the local IRB. All investigators were trained for the diagnosis, history review, disease activity evaluation, laboratory examinations, data input, and sample collection by local or nationwide training programs. This ongoing registry had recruited 2170 Chinese SLE patients who fulfilled the SLE classification criteria revised by the American College of Rheumatology (ACR) in 1997 [9] during the period between April 2009 and February 2010. Patients were required to fulfill at least 4 of the following 11 criteria: (1) malar rash; (2) discoid rash; (3) photosensitivity; (4) oral or nasopharyngeal ulceration; (5) nonerosive arthritis involving 2 or more peripheral joints; (6) pleuritis or pericarditis; (7) nephropathy: persistent proteinuria > 0.5 grams per day or cellular casts; (8) neurologic involvement: seizures or psychosis in the absence of offending drugs or known metabolic derangements; (9) hematologic involvement: hemolytic anemia with reticulocytosis or leukopenia (<4,000/mm^3 on ≥2 occasions) or lymphopenia (<1,500/mm^3 on ≥2 occasions) or thrombocytopenia (<100,000/mm^3) in the absence of offending drugs; (10) immunologic disorder: antibody to native double-stranded DNA in abnormal titer or presence of antibody to Sm nuclear antigen or positive finding of antiphospholipid antibodies; (11) positive antinuclear antibody. We confirmed positive finding of antiphospholipid antibodies by an abnormal serum level of IgG or IgM anticardiolipin antibodies, an abnormal serum level of anti-β2 glucoprotein I, or a positive test result for lupus anticoagulant. In this study, we analyzed baseline data of 2104 patients (including 190 male and 1914 female patients) (Table 1).

2.2. Methods. Each center of the CSTAR has provided uniform evaluations and recorded data following the same protocol and operational procedures. Clinical manifestations and systemic involvement of SLE patients were collected, evaluated, and the relevant data were entered into the online CSTAR registry database. Pulmonary arterial hypertension (PAH) was defined as a resting systolic PAP (PASP) ≥40 mmHg estimated by echocardiography [10] without chronic lung conditions, cardiac valvular, or cardiomyopathic complications. Interstitial lung disease (ILD) was detected by chest X-ray or computerized tomography without infective infiltrations. Patients who had ILD with known causes or PAH were excluded when the association between autoantibodies and ILD in this study was analyzed.

The autoantibodies were measured at the local labs of each center, including anti-nuclear antibody (ANA), anti-double stranded DNA (anti-dsDNA) antibody, anti-Sm antibody, anti-ribosomal RNA-protein (anti-rRNP) antibody, anti-SSA antibody, anti-SSB antibody, anti-u1 small-nuclear RNA-protein (anti-RNP) antibody, and anti-phospholipid (APL) antibody. Most centers detected ANA and anti-dsDNA antibody using immunofluorescence assay with Hep-2 cell line and anti-extractable nuclear antigen (ENA) antibody (including anti-Sm, anti-SSA, anti-SSB, anti-RNP, and anti-rRNP antibodies) was tested with immunoblotting assay. The APL antibody was tested using enzyme-linked immunosorbent assay (anticardiolipin and anti-β2 glucoprotein I antibody) or dilute Russell viper venom test (lupus anticoagulant) when anti-phospholipid syndrome was suspected but these tests were not mandatory.

2.3. Statistical Analysis. Statistical analysis was performed by using SPSS 17.0 for WINDOWS. Positivity of autoantibodies in SLE patients with different clinical manifestations was expressed as patient number with percentage (%) in brackets (Table 4). Chi-square tests were performed to detect the associations between clinical manifestations and autoantibody patterns. Since there was no expected frequency <5, Fisher's exact test was not used. Cluster analysis with Ward's method was performed to investigate the relationship between autoantibodies. All tests of significance were two-sided and a P value of < 0.05 was considered to be statistically significant.

3. Results

The autoantibody profile of this study included the presence of ANA in 2063 (98.1%), anti-dsDNA antibody in 699 (33.2%), anti-Sm antibody in 350 (16.6%), anti-RNP antibody in 189 (8.9%), anti-SSA antibody in 497 (23.6%), anti-SSB antibody in 224 (10.7%), and anti-rRNP antibody in 255 (12.7%) cases. APL antibody was tested in 937 patients with a positivity of 44.1% (414/937). 199 patients (9.5%) were

Table 1: The baseline characteristics of 2104 SLE patients from CSTAR cohort study.

		%
Female	1914	91.0
Male	190	9.0
Age at onset (years)	29.2 ± 12.1 (range 1.4~68.9)	
Age at diagnosis (years)	30.3 ± 12.3 (range 4~77)	
Age at entry (years)	32.7 ± 12.7 (range 5~78)	
Disease duration (months)	41.9 ± 58.8 (range 1~468)	
SLE disease activity index at entry		
0~4	532	25.3
5~9	587	27.9
10~14	591	28.1
>14	394	18.7

demonstrated to have both anti-SSA antibody and anti-SSB antibody and 142 patients (6.7%) have anti-Sm antibody and anti-RNP antibody, simultaneously (Table 2).

Clinical manifestations found in our study included malar rash in 1009 (47.9%), discoid skin lesions in 118 (5.6%), photosensitivity in 526 (25.0%), oral ulcer in 466 (22.1%), arthritis in 1147 (54.5%), serositis in 345 (16.4%), nephropathy in 988 (47.4%), hematological involvement, including leukocytopenia, hemolytic anemia, and thrombocytopenia, in 1181 (56.1%), and neurological involvement (neuropsychological lupus) in 101 (4.8%) patients. The prevalence of ILD and PAH was 4.2% (86/2024) and 3.8% (74/1934), respectively in this registry database (Table 3).

The association analysis between clinical manifestations and autoantibodies revealed that there were associations between anti-Sm antibody ($P < 0.001$), anti-rRNP antibody ($P < 0.05$), and malar rash; between anti-dsDNA antibody and nephropathy; between anti-RNP antibody, anti-SSA antibody and pulmonary arterial hypertension (PAH); and between anti-SSB antibody and hematological involvement ($P < 0.05$). Significant associations were also found between anti-dsDNA antibody and a lower prevalence of photosensitivity and between anti-SSA antibody and a lower prevalence of nephropathy ($P < 0.05$). APL antibody was associated with hematologic involvement, interstitial lung disease (ILD), and a lower prevalence of oral ulcerations ($P < 0.05$) (Table 4).

Using cluster analysis, we identified five clusters of antibodies. Cluster 1 consisted of antibodies to Sm and RNP and cluster 2 consisted of antibodies to SSA and SSB. Clusters 3, 4, and 5 consisted of antibodies to ribosomal P, dsDNA, and APL, respectively (Figure 1).

4. Discussion

Diffuse connective tissue diseases (CTDs) are characterized by the presentation of specific profiles of autoantibodies, and SLE is the prototype of diffuse CTDs, which could involve almost all systems [1]. The typical pathological feature of SLE is systemic vasculitis with immune complex deposition [11].

Table 2: The profile of autoantibodies in 2104 SLE patients from CSTAR cohort study.

	Patients number	Positivity (%)
Anti-nuclear antibody (ANA)	2063	98.1
Anti-double stranded DNA (anti-dsDNA) antibody	699	33.2
Anti-Sm antibody	350	16.6
Anti-SSA antibody	497	23.6
Anti-SSB antibody	224	10.7
Anti-u1 small-nuclear RNA-protein (anti-RNP) antibody	189	8.9
Anti-ribosomal RNA-protein (anti-rRNP) antibody	255	12.7
Anti-phospholipid (APL) antibody	414/937[$]	44.1
Anti-SSA and anti-SSB antibody positive simultaneously	199	9.5
Anti-Sm and anti-RNP antibody positive simultaneously	142	6.7

[$] Actually detected number of patients.

Table 3: The profile of clinical manifestations in 2104 SLE patients from CSTAR cohort study.

	Patients number	Positivity (%)
Malar rash	1009	47.9
Discoid lesions	118	5.6
Photosensitivity	526	25.0
Oral ulcerations	466	22.1
Arthritis	1147	54.5
Serositis	345	16.4
Nephropathy	998	47.4
Hematological involvement (hematocytopenia)	1181	56.1
Neurological involvement	101	4.8
Interstitial lung disease	86/2024[$]	4.2
Pulmonary arterial hypertension	74/1934[$]	3.8

[$] Actually detected number of patients.

It is rational to propose that some autoantibodies may be associated with specific clinical features of SLE.

Anti-dsDNA has been proven to be a pathogenic autoantibody in SLE and has been reported to be associated with renal damage, leukopenia, anemia, and urine cellular casts in SLE patients [6, 7, 12]. Chien et al. discovered that there were associations between anti-dsDNA antibodies and multiple clinical manifestations of SLE patients, such as Raynaud's phenomenon, photosensitivity, arthritis, hypocomplementemia, thrombocytopenia, proteinuria, and serositis, but with a small number of patients (80 patients) [13]. Our study confirmed that anti-dsDNA was associated with nephropathy (Table 4) which was also reported by

Lu et al. and Alba et al. [12, 14]. The association between anti-dsDNA antibody and a lower prevalence of photosensitivity observed in our study was contradictory to Smikle et al. [4]. The difference may be due to different ethnical background and sample size.

As a marker autoantibody of SLE, anti-Sm antibody was found to be associated with malar rash, discoid rash, pericarditis, and leukopenia in studies by Tang et al. and Lu et al. [7, 12] However, the pathogenic characteristics of anti-Sm antibody were controversial [6, 12, 14, 15]. Our study also revealed the association of anti-Sm antibody with malar rash, and malar rash is a characteristic skin lesion of SLE patients. But the association between anti-Sm antibody and other organ damage could not be detected in our study (Table 4).

Anti-RNP antibody was thought to be related to Raynaud's phenomenon and PAH by many physicians. The association between anti-RNP and Raynaud's phenomenon was confirmed by Hoffman et al. and Tang et al. [6, 7]. Both Raynaud's phenomenon and anti-RNP antibody are considered as risk factors for PAH associated with CTDs and represent the presence of vasculopathy [16]. Anti-RNP antibody was also found to be associated with photosensitivity [7], lymphopenia [12], and leukopenia [6]. In our study, the Raynaud's phenomenon was not included in the clinical manifestations analysis, but the association between anti-RNP antibody and PAH in patients with SLE was discovered (Table 4).

Anti-SSA and anti-SSB antibody are frequently found in SLE patients with the positivity ranged from 34% to 83% in different reports [8, 17] and higher prevalence of anti-SSA/SSB antibody could be observed in SLE patients associated with secondary Sjogren's syndrome [18]. Anti-SSA antibody was found to be associated with neonatal heart block [19], xerophthalmia/xerostomia, and photosensitivity [20]. Anti-SSB body was found to be associated with hematological disorder, proteinuria, malar rash [12], and pericarditis [6]. In this study, we found the associations between anti-SSA antibody and PAH, between anti-SSB antibody and hematological involvement, and between anti-SSA antibody and a lower prevalence of nephropathy (Table 4). This study is the first report that anti-SSA antibody might be a predictor of PAH in SLE patients according to our knowledge. Since we have known that anti-SSA antibody is one of the diagnostic criteria for Sjogren's syndrome [21] and PAH is also a rare manifestation of patients with primary Sjogren's syndrome [22], we propose that more attention should be paid to screen for PAH in SLE patients with anti-SSA antibody. But further studies are needed to clarify this association in the future. The association between anti-SSA antibody and a lower prevalence of lupus nephritis was reported by Chien and coresearchers in a small sample size study [13] and Tapanes et al. found that anti-SSA antibody may correlate with favorable prognosis of lupus nephritis [23]. But Vila et al. found the opposite results in 201 Puerto Ricans patients with SLE [24]. We confirmed the association between anti-SSA antibody and a lower prevalence of nephropathy in Chinese SLE patients and this association may suggest a protective role of anti-SSA antibody in lupus nephritis. The hematological disorder in SLE patients with positive anti-SSB antibody was primarily thrombocytopenia in our study and the association between anti-SSB antibody and thrombocytopenia was reported by Unal et al. in a case report [25]. Large sample studies are needed to clarify the real association between the hematological disorders and anti-SSB antibody.

Anti-rRNP antibody is regarded as a specific autoantibody of SLE [26] and it is thought to be associated with neuropsychological manifestations of SLE patients [27]. This relationship was not proven in our study, what may be due to the small amount of patients with neuropsychological manifestations in our registry. The association of anti-rRNP antibody and malar rash was found in our study (Table 4), which was consistent with anti-Sm antibody.

The APL antibody was tested in 937 patients when antiphospholipid syndrome was suspected in our cohort study. The association between APL antibody and hematological involvement (mainly thrombocytopenia) was deductible since phospholipid is an innate component of blood cells. McClain and coresearchers have found that APL antibody appeared prior to diagnosis of SLE and this group of autoantibodies are associated with many SLE features, including malar rash, discoid lesions, photosensitivity, renal disorder, neurological disorder, hemolytic anemia, and thrombocytopenia [28]. Saches and coresearchers have demonstrated that APL antibody is associated with spontaneous abortion, thrombocytopenia, livedo reticularis, and a positive direct Coombs' test in SLE patients [29]. The association between APL antibody and ILD found in our study may be related to the microvessel injuries resulted from microemboli or immune-complex deposition (Table 4). Kanakis et al. have reviewed the pulmonary manifestations in patients with antiphospholipid syndrome including fibrosing alveolitis [30]. We confirmed the association between antiphospholipid antibody and ILD, which is a rare feature of SLE. Neurological involvement in SLE patients is thought to be correlated with APL antibody [31, 32], but previous studies have not shown this association [28, 29], perhaps due to the low incidence of neuropsychological lupus in SLE patients. We demonstrated a tendency ($P = 0.061$) of association between APL antibody and neurological involvement in our study (Table 4). Further studies with more patients are needed.

Using cluster analysis, we identified five clusters of autoantibodies. Antibodies to Sm and RNP clustered together early. Cluster 2 consisted of antibodies to SSA and SSB. The other clusters consisted of individual antibodies to ribosomal P (rRNP), dsDNA, and APL, respectively (Figure 1). Our result is in accordance with previous studies [6, 33].

We summarize the associations with statistical significance between specific autoantibodies and clinical manifestations revealed by different study groups in Table 5. Most of our findings are consistent with studies in the literature, but the associations between anti-RNP antibody and PAH; between anti-SSA and PAH; and between APL antibody and ILD were first discovered by our study. We always repeated echocardiography if PAH was suspected in the first echocardiogram examination. Right heart catheter (RHC) is not feasible for general screening and repeated

Table 4: Associations between specific autoantibodies and clinical manifestations of SLE [patient number (%)].

	Anti-dsDNA		Anti-Sm		Anti-RNP		Anti-SSA		Anti-SSB		Anti-rRNP		APL	
	Positive	*P*	Positive	*P*	Positive	*P*	Positive	*P*	Positive	*P*	Positive	*P*	Positive	*P*
Patients number	699 (100)		350 (100)		189 (100)		497 (100)		224 (100)		255 (100)		414 (100)	
Malar rash	315 (45.06)	0.061	201 (57.43)	<0.001*	91 (48.15)	0.956	241 (48.49)	0.785	105 (46.88)	0.732	151 (59.22)	0.004*	187 (45.17)	0.275
Discoid lesions	31 (4.43)	0.099	22 (6.29)	0.546	13 (6.88)	0.426	25 (5.03)	0.522	11 (4.91)	0.631	13 (5.10)	0.855	20 (4.83)	0.540
Photosensitivity	150 (21.46)	0.008*	95 (27.14)	0.311	47 (24.87)	0.965	132 (26.56)	0.358	67 (29.91)	0.073	69 (27.06)	0.752	96 (23.19)	0.509
Oral ulcerations	151 (21.6)	0.671	87 (24.86)	0.181	42 (22.22)	0.980	108 (21.73)	0.797	48 (21.43)	0.784	48 (18.82)	0.395	74 (17.87)	0.035*
Arthritis	399 (57.08)	0.095	193 (55.14)	0.796	106 (56.08)	0.650	268 (53.92)	0.762	119 (53.13)	0.658	146 (57.25)	0.765	236 (57.00)	0.289
Serositis	119 (17.02)	0.584	55 (15.71)	0.705	31 (16.40)	0.999	89 (17.91)	0.298	46 (20.54)	0.077	35 (13.73)	0.428	61 (14.73)	0.996
Nephropathy	355 (50.79)	0.030*	155 (44.29)	0.196	91 (48.15)	0.837	216 (43.46)	0.042*	102 (45.54)	0.547	120 (47.06)	0.817	191 (46.14)	0.848
Hematological involvement	403 (57.65)	0.321	196 (56.00)	0.957	108 (57.14)	0.769	278 (55.94)	0.920	141 (62.95)	0.030*	137 (53.73)	0.542	273 (65.94)	<0.001*
Neurological involvement	26 (3.72)	0.102	18 (5.14)	0.743	12 (6.35)	0.296	21 (4.23)	0.493	13 (5.80)	0.457	16 (6.27)	0.335	32 (7.73)	0.061
ILD	29/675[$] (4.30)	0.941	18/326[$] (5.52)	0.214	11/181[$] (6.08)	0.201	21/481[$] (4.37)	0.884	12/209[$] (5.74)	0.259	12/250[$] (4.80)	0.940	29/398[$] (7.29)	<0.001*
PAH	24/661[$] (3.63)	0.747	16/322[$] (4.97)	0.242	12/180[$] (6.67)	0.037*	28/469[$] (5.97)	0.005*	11/205[$] (5.37)	0.224	5/237[$] (2.11)	0.151	15/387[$] (3.88)	0.714

P: *P* value; Anti-dsDNA: Anti-double stranded DNA antibody; Anti-Sm: Anti-Sm antibody; Anti-SSA: Anti-SSA antibody; Anti-SSB: Anti-SSB antibody; Anti-RNP: Anti-u1 small-nuclear RNA-protein antibody; Anti-rRNP: Anti-ribosomal RNA-protein antibody; APL: Anti-phospholipid antibody; PAH: pulmonary arterial hypertension; ILD: interstitial lung disease.

[$]Actually detected number of patients.

*$P < 0.05$.

Table 5: Associations with statistical significance between specific autoantibodies and clinical manifestations in SLE patients.

Author	Number of patients	Geographical area	Anti-dsDNA	Anti-Sm	Anti-RNP	Anti-SSA	Anti-SSB	Anti-rRNP	APL
Chien et al. [13]	80	Asia, China	Raynaud's phenomenon Photosensitivity Arthritis Thrombocytopenia Hypocomplementemia Proteinuria Serositis			Photosensitivity Anemia			
McClain et al. [28]	130	United states							Malar rash discoid lesions photosensitivity renal disorder neurological disorder hemolytic anemia thrombocytopenia
Vila et al. [24]	201	Puerto Ricans	Vasculitis Pericardial effusion Renal involvement Anaemia Leukocytopenia Lymphocytopenia Thrombocytopenia	Skin ulcerations Elevated liver enzymes Renal involvement Thrombocytopenia		Discoid rash Serositis Pneumonitis Elevated liver enzymes Hemolytic anaemia Leukocytopenia Lymphocytopenia			
Hoffman et al. [6]	289	Europe, Belgium	Urine cellular casts		Raynaud's phenomenon Leukocytopenia	Xerostomia	Xerostomia Pericarditis		
Tang et al. [7]	917	Asia, China	Renal disorder Leukocytopenia Anemia	Malar rash Discoid rash Pericarditis Leukocytopenia	Raynaud's phenomenon Photosensitivity				
Lu et al. [12]	1803	United states	Renal disease Urine cellular casts	Leukocytopenia		Hematological disorder Lymphocytopenia	Hematological disorder Proteinuria Malar rash		
CSTAR	2104	Asia, China	Photosensitivity Nephropathy	Malar rash	PAH	Nephropathy PAH	Hematological disorder	Malar rash	Oral ulceration hematological disorder ILD

ANA: Anti-nuclear antibody; Anti-dsDNA: Anti-double stranded DNA antibody; Anti-Sm: Anti-Sm antibody; Anti-SSA: Anti-SSA antibody; Anti-SSB: Anti-SSB antibody; Anti-RNP: Anti-u1 small-nuclear RNA-protein antibody; Anti-Rrnp: Anti-ribosomal RNA-protein antibody; APL: Anti-phospholipid antibody; PAH: pulmonary arterial hypertension; ILD: interstitial lung disease.

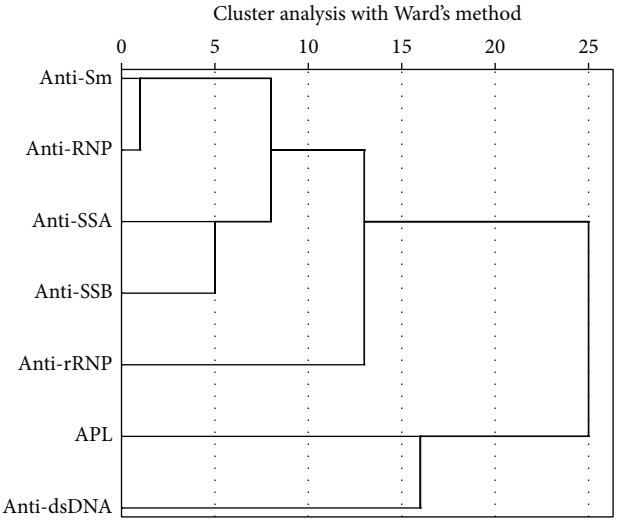

Figure 1: Result of cluster analysis with Ward's method in SLE patients. Five clusters of antibodies were identified. Cluster 1 consisted of antibodies to Sm and RNP and cluster 2 consisted of antibodies to SSA and SSB. Clusters 3, 4, and 5 consisted of antibodies to ribosomal P, dsDNA, and APL, respectively.

measurements due to its invasive characteristic but RHC is used for confirming the diagnosis of PAH according to the guideline. As far as we know, most of the 74 patients with a preliminary diagnosis of PAH identified in our study have been referred for confirmatory RHC, but these results were not recorded in the registry. This is a limitation of our study [34].

5. Conclusion

As the largest registry cohort study in China, CSTAR has already disclosed some clinical profiles of Chinese SLE patient [8, 34]. Confirmation of the associations between clinical manifestations and specific autoantibodies found in our study can help physicians to understand the features of SLE patients better, especially in China. A thorough screening of ANA and anti-ENA antibodies when the diagnosis of SLE is established can help us to predict organ damage. We could focus on the specific autoantibody-related vital organ complications (e.g., PAH) in the follow-up of SLE patients. We suggest that repeating echocardiography annually may help to discover PAH at the early stage in SLE patients with anti-RNP and/or anti-SSA antibody. Early diagnosis of PAH in patients with SLE is important for initiating effective interventions to prevent malignant outcomes (e.g., heart failure).

Abbreviations

ANA: Anti-nuclear antibody
ENA: Extractable nuclear antigen
Anti-dsDNA: Anti-double stranded DNA antibody
Anti-Sm: Anti-Sm antibody
Anti-SSA: Anti-SSA antibody
Anti-SSB: Anti-SSB antibody
Anti-RNP: Anti-u1 small-nuclear RNA-protein antibody
Anti-rRNP: Anti-ribosomal RNA-protein antibody
APL: Anti-phospholipid antibody
PAH: Pulmonary arterial hypertension
ILD: Interstitial lung disease.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Jing Li, Xiaomei Leng, and Zhijun Li contributed equally to this study. All authors made substantial contributions to conception and design. Dr. Jing Li, Dr. Xiaomei Leng, and Dr. Zhijun Li performed the interpretation of data and wrote the paper. Dr. Jiuliang Zhao performed the analysis of data. Dr. Mengtao Li and Dr. Xin-Ping Tian revised the paper critically. Dr. Xiaofeng Zeng gave final approval of the version to be submitted. All authors read and approved the final paper.

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Research Article

Antilymphocyte Antibodies in Systemic Lupus Erythematosus: Association with Disease Activity and Lymphopenia

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Purpose. We analyzed the prevalence, clinical correlation, and the functional significance of ALA in patients with systemic lupus erythematosus (SLE). *Methods*. ALA IgG was detected by indirect immunofluorescence in the serum of 130 SLE patients, 75 patients with various rheumatic diseases, and 45 healthy controls (HC). *Results*. The sensitivity and specificity of ALA IgG in SLE were 42.3% and 96.7%, respectively. ALA was observed in 55.6% (50/90) of patients with lymphopenia, which was significantly higher than in patients with normal lymphocytes (5/40, 12.5%; $P < 0.001$). Patients with active SLE showed higher ALA positivity (60.9%) than those with inactive disease (24.2%; $\chi^2 = 17.925$; $P < 0.001$). ALA correlated significantly with hypocomplementemia, anti-dsDNA antibodies, and higher SLEDAI scores. The incidences of ALA in SLE patients who were seronegative for anti-dsDNA, anti-Sm, or both antibodies were 32.9% (26/79), 41.0% (43/105), and 32.4% (22/68), respectively. The ALA-positive group also had higher incidences of neuropsychiatric SLE (NPSLE) and lupus nephritis (LN). In multivariate analyses, ALA was independently associated with lymphopenia, higher SLEDAI scores, and increased risk for LN. ALA titers significantly decreased as clinical disease was ameliorated following treatment. *Conclusions*. ALA occurred more frequently in patients with active SLE and was independently associated with lymphopenia, disease activity, and LN.

1. Introduction

Lymphopenia is a common clinical manifestation in systemic lupus erythematosus (SLE) and is one of the diagnostic criteria, according to the American College of Rheumatology (ACR) classification [1]. Lymphopenia was observed in 62% of adult patients at the diagnosis of SLE [2]. The cumulative percentage of the occurrence of lymphopenia over the course of the disease reached over 90% in an adult series [2]. In addition to its clinical use as a diagnostic marker, lymphopenia is associated with disease activity and organ damage [3, 4]. Studies have also suggested that lymphopenia is a risk factor for carotid intima-media thickness in juvenile-onset SLE [5].

Because lymphopenia is a common manifestation in SLE, people have long been interested in antibodies against lymphocytes. Lymphocytotoxic antibodies (LCA) were found in the great majority of patients with SLE [6, 7]. The standard method for the detection of LCA is a microcytotoxicity test. However, subtle differences in the protocol, such as incubation/isolation temperature, method of target cell isolation, and serum dilution, can result in result variability, and this has created many controversies in the literature. A practical indirect immunofluorescence test, for ALA, was developed to overcome the shortcomings of LCA. LCA was shown to be cold-reactive and to detect IgM exclusively [6, 8, 9]. However, Agnello suggested that the IgG type may be more important functionally [10] and more effective at physiological temperatures.

Despite this, there has been little research investigating the role of ALA IgG in SLE. The causal relationship between ALA and lymphocyte function is also largely unexplored. In the present study, we detected ALA IgG and analyzed

the possibility of using ALA IgG as a biomarker for disease activity in SLE. We also explored the relationship between lymphopenia and ALA.

2. Materials and Methods

2.1. Patients. In total, 130 Chinese SLE patients who attended to the Department of Rheumatology and Immunology, Peking University People's Hospital, were enrolled. All patients were on stable doses of glucocorticoids in the previous month and did not used immunosuppressants in the previous 6 months. Serum samples were obtained from the patients. All patients fulfilled the 1997 revised American College of Rheumatology SLE criteria [1].

Also, 75 patients with other autoimmune diseases were used as a disease control group. There were 16 Sjögren's syndrome (SS), 21 rheumatoid arthritis (RA), 16 ankylosing spondylitis (AS), 5 dermatomyositis/polymyositis (DM/PM), 5 undifferentiated connective tissue diseases (UCTD), 7 systemic sclerosis (SSc), and 5 osteoarthritis (OA) cases. In these control patients, there were 56 females and 19 males (female : male = 2.95 : 1). Healthy control (HC) serum samples were obtained from 45 blood donors. All sera were kept at −20°C.

The protocol for the study was approved by the Ethical Committee of Peking University People's Hospital (FWA00001384).

2.2. Methods

2.2.1. Detection of Antilymphocyte Antibody (ALA). Serum ALA was assayed using an indirect immunofluorescence test kit according to the manufacturer's protocol (EUROIMMUN, Germany).

2.2.2. Identification of ALA. The identification of ALA included two steps to ensure the specificity. First, ANCA were detected by an indirect immunofluorescence test (EUROIMMUN) to exclude cross-reactivity. Second, Biochip slides were incubated with RNase-free DNase RQ1 (1 U/μL; TaKaRa Biotechnology, Dalian, China) at room temperature. Control slides were treated with trypsin (0.125 μg/μL, Dingguo, Beijing) for 30 min at 37°C. Then, 5 μL DNase RQ1 was added to serum samples in a final volume of 50 μL and incubated for 60 min at room temperature. Also, 5 μL enzyme-free buffer was used as a control. The reaction was stopped by adding 5 μL 0.5 Methylenediaminetetraacetic acid (EDTA). After washing the slides twice in PBS, subsequent steps were performed according to the manufacturer's protocol.

2.2.3. Clinical and Laboratory Parameters. Clinical and laboratory features of SLE patients were recorded. Lymphopenia was defined according to the ACR criteria (<1.5 × 10^9/L) and was scored only if physicians determined that it was attributed to SLE and not to medications or other causes. Leucopenia was defined as a white blood cell (WBC) count <4 × 10^9/L. The "systemic lupus erythematosus disease activity index" (SLEDAI) was used to assess disease activity. A SLEDAI score > 8 was defined as active lupus, as described previously [11].

Table 1: Demographic, clinical and laboratory features of SLE patients.

Characteristics	
Female, *n* (%)	122 (93.8)
Age, mean ± SD years	33.3 ± 12.4
Disease duration, median (interquartile range)	2 (0.8–5)
SLEDAI, mean ± SD	9.8 ± 5.8
Clinical features, *n* (%)	
Lupus nephritis	64 (49.2)
NPSLE	13 (10)
Arthralgia	45 (34.6)
Serositis	15 (11.5)
Anti-dsDNA positive, *n* (%)	50 (38.5)
ALA, *n* (%)	55 (42.3)

2.3. Statistical Analysis. Data with a normal distribution are expressed as the mean ± SD. Nonnormally distributed data are expressed as the median and interquartile range. Categorical variables were analyzed using the χ^2 test or Fisher's exact test. Student's t-test or analysis of variance (ANOVA) was used for continuous data and the Mann-Whitney U-test was used for nonnormally distributed data. The ALA titers before and after treatment were compared using Wilcoxon's signed rank test. Multivariate analysis was performed to investigate the relationship between ALA, lymphopenia, the development of LN or NPSLE and SLEDAI scores, adjusting for gender, age, and disease duration. Statistical significance was defined as $P < 0.05$ (two-tailed). All statistical analyses were performed using the SPSS software (ver. 16.0).

3. Results

Under microscopic examination, ALA was shown as a fluorescence of the cytoplasm or as a linear annular fluorescence of the lymphocyte cell membrane (Figure 1(a)). For serum without ALA, there was no fluorescence in either the cytoplasm or around the cell membrane (Figure 1(b)). In the identification test, there was no fluorescence in the cytoplasm or linear annular fluorescence in neutrophils or granulocytes on slides. The fluorescence pattern and intensity did not change after pretreatment with RNase-free DNase RQ1 (Figure 1(c)), whereas there was no fluorescence in slides pretreated with trypsin (Figure 1(d)), indicating that the ALA antigen was a protein.

Of the 130 SLE patients, 55 (42.3%) were positive for ALA. No healthy control was positive for ALA ($P < 0.001$; Table 1). The prevalence in other rheumatic diseases was significantly lower at 5.6% (4/72, $P < 0.001$, Figure 2). The sensitivity and specificity of ALA for the diagnosis of SLE were 42.3% and 96.7%, respectively.

The positive rate of ALA in the lymphopenia group was much higher than in the normal lymphocyte group (55.6% versus 12.5%; $P < 0.001$). The prevalences of lupus nephritis

Table 2: The correlation of ALA and clinical manifestations in SLE.

Clinical Features	ALA negative ($N = 75$)		ALA positive ($N = 55$)		χ^2	P values
	n	%	n	%		
LN	22	29.3	42	76.4	28.080	**0.000**※
NPSLE	3	4.0	10	18.2	7.091	**0.008**※
Skin Rash	35	46.7	27	49.1	0.075	0.785
Photosensitivity	8	10.7	2	3.6	1.330	0.189
Oral Ulcer	10	13.3	8	14.5	0.039	0.843
Arthralgia	29	38.7	16	29.1	1.286	0.257
Serositis	7	9.3	8	14.5	0.845	0.358
Raynaud Phenomenon	6	8.0	6	10.9	0.320	0.571
Alopecia	21	28	18	32.7	0.338	0.561
Myositis	5	6.7	1	1.8	1.694	0.193

※$P < 0.05$.

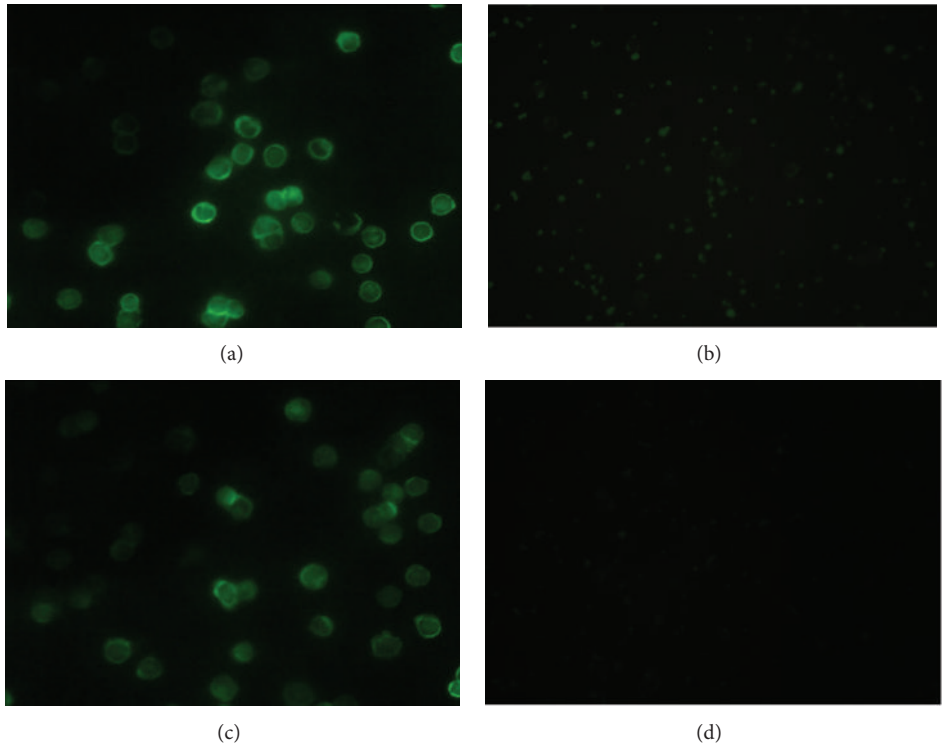

Figure 1: ALA immunofluorescence patterns (×400). (a) ALA-positive pattern with fluorescence in the cytoplasm or a linear annular fluorescence around the lymphocyte cell membrane. (b) ALA-negative pattern with the absence of fluorescence in the cytoplasm or the lymphocyte cell membrane. (c) Immunofluorescence patterns on lymphocytes pretreated with DNase. (d) Immunofluorescence patterns on lymphocytes pretreated with trypsin. This result indicated that the ALA antigen was a protein.

(76.4%) and NPSLE (18.2%) in ALA-positive SLE patients were significantly higher than in ALA-negative SLE patients (29.3% and 4%, resp.; $P < 0.05$) (Table 2). The SLEDAI score of the ALA-positive group was 11.84 ± 5.36, compared with 8.33 ± 5.71 in the negative group ($P < 0.05$). Using multivariate logistic regression, adjusting for gender, age, and disease duration, ALA was independently associated with lymphopenia (odd ratio (OR) 6.034, 95% confidence interval (CI) 1.385–26.301; $P = 0.017$), disease activity (OR 3.713, 95% CI 1.560–8.835; $P = 0.003$), and increased risk for LN (OR 5.873, 95% CI 2.523–13.672; $P < 0.001$), but not for NPSLE (OR 4.495, 95% CI 0.969–20.865; $P = 0.055$).

Serum complement (C3, C4) levels were much lower in ALA-positive versus ALA-negative patients ($P < 0.05$; Table 3). The frequencies of ANA and anti-dsDNA antibodies were significantly higher in ALA-positive SLE patients (94.5% and 50.9%) than in the ALA-negative group (80% versus 29.3%, $P < 0.05$) (Table 3).

ALA was also found in some anti-dsDNA and anti-Sm-negative patients. The positive rates of ALA were 32.9%,

Table 3: The relation between ALA and other laboratory parameters in SLE.

Laboratory parameters	ALA negative ($N = 75$)		ALA positive ($N = 55$)		χ^2	P values
	n	%	n	%		
ANA	60	80	52	94.5	5.628	**0.018**※
Anti-dsDNA	22	29.3	28	50.9	6.241	**0.012**※
Anti-SSA	20	26.7	22	40.0	2.579	0.108
Anti-SSB	5	6.7	6	10.9	0.737	0.391
Anti-RNP	14	18.7	11	20.0	0.036	0.849
Anti-Sm	10	13.3	7	12.7	0.010	0.919
ESR Elevation	50	66.7	45	81.8	3.702	0.054
CRP Elevation	27	36	17	30.9	0.367	0.544
C3 Decrease	58	77.3	54	98.2	11.562	0.001※
C4 Decrease	51	68	48	87.3	6.490	0.011※
IgA Elevation	13	17.3	14	25.5	1.272	0.259
IgG Elevation	34	45.3	29	52.7	0.695	0.405
IgM Elevation	5	6.7	5	9.1	0.263	0.608
Leucopenia	31	41.3	32	58.2	3.606	0.058
Thrombocytopenia	16	21.3	8	14.5	0.971	0.324

※$P < 0.05$.

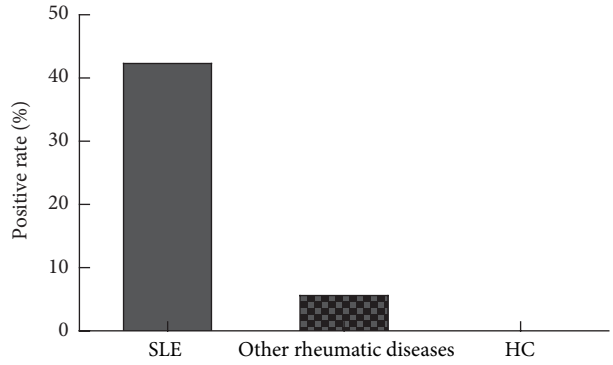

Figure 2: Prevalence of ALA in patients with SLE. The positive rate of ALA in patients with SLE was significantly higher than the other conditions in the control group (4/75, 5.6%): DM/PM (1/5, 20%), SS (2/16, 12.5%), RA (1/21, 4.8%), UCTD (0/5, 0%), SSc (0/7, 0%), OA (0/5, 0%), AS (0/16, 0%), and HC (0/45, 0%), respectively ($P < 0.001$).

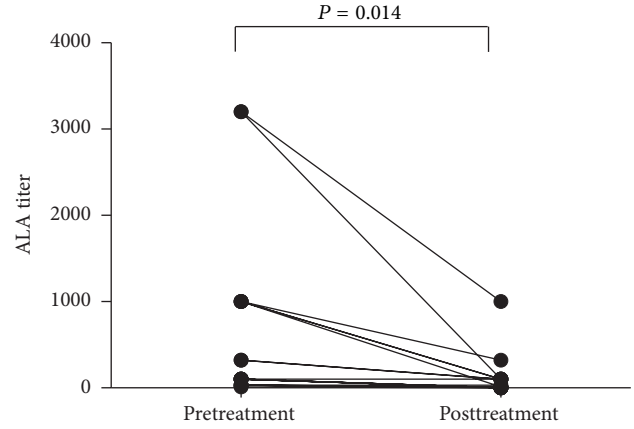

Figure 3: ALA titers before and after treatment in 20 patients with SLE. The ALA titers decreased significantly in accordance with clinical amelioration of disease following treatment ($P = 0.014$).

41.0%, and 32.4% in anti-dsDNA negative, anti-Sm negative, and double-negative SLE patients, respectively.

ALA titers decreased significantly in accordance with disease amelioration following treatment in a subgroup of 20 patients with SLE (Figure 3). The SLEDAI scores in these 20 patients also decreased from 15.8 ± 6.2 to 3.6 ± 3.0 ($P < 0.001$).

4. Discussion

Our study demonstrated that the sensitivity and specificity of ALA IgG in SLE were 42.3% and 96.7%, respectively. ALA was independently associated with lymphopenia. The SLEDAI scores and the prevalences of LN and NPSLE were significantly higher in ALA-positive SLE patients than in ALA-negative SLE patients. ALA was also associated with disease activity and LN. Multivariate analysis revealed that ALA was independently associated with disease activity and increased risk for LN. ALA titers decreased significantly in accordance with disease amelioration and monitoring the titer of this antibody may be helpful for predicting disease flares.

As suggested by Magalhães et al., LCA in SLE is associated with disease activity, regardless of the presence of neuropsychiatric manifestations [12]. In the present study, we also found that ALA was associated with disease activity parameters, such as hypocomplementemia, anti-dsDNA antibody, and SLEDAI scores, confirming that ALA is a meaningful biomarker for disease activity (Table 3). Furthermore, ALA was related to anti-dsDNA antibody; however, it was also seen in 32.9% of anti-dsDNA-negative SLE patients. Thus,

ALA may be a better or supplementary parameter of disease activity.

ALA is believed to act in both cryoprecipitate formation and the development of organ damage [13, 14]. Our results are consistent with previous studies showing that ALA was correlated with organ involvement, such as LN, indicating that ALA is a predictor for poor prognosis and may play a role in the pathogenesis of SLE. Osman and Swaak's study showed an overlap between ALA and anti-β2-microglobulin [15]. ALA may have an impact on T cells as well as on B-cell function and play a role in LN [16]. Although complete information concerning the molecules with which ALA interacts is not yet available, possible target antigens of ALA include CD45, T-cell receptors, β2-microglobulin, and HLA I/HLA II antigens [17].

To date, no direct relationship between ALA and lymphopenia has been reported. In our study, ALA was present in more than half of the SLE patients with lymphopenia. Of the patients with ALA, 90.9% had lymphopenia. In a multivariate analysis, ALA was independently associated with lymphopenia. The results suggest that ALA might be one of the reasons for lymphopenia. Possible mechanisms include the depletion of circulating T cells and ALA may have the capacity for direct actions on target cells, including complement-dependent cytotoxicity and ADCC, modulation of surface antigens, and up- or downregulation of various cell functions in the immune response [15]. Other possible explanations for lymphopenia have been proposed. Several other autoantibodies, such as anti-SSA antibody, anti-snRNP antibody, and anti-dsDNA antibody, may have lymphocytotoxic properties. Another contributing factor to lymphopenia involves defective CD95/Fas systems [18] and diminished expression of complement regulatory proteins (CD55 and CD59) [19]. Despite these hypotheses, our results indicate that ALA plays a role in the pathogenesis of lymphopenia in SLE patients.

5. Conclusions

In conclusion, ALA IgG is a good parameter of disease activity and is associated with LN in SLE. The presence of ALA may be important in the mechanism of lymphopenia in SLE.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Rong Mu contributed equally to this paper.

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Research Article

Anti-Cyclic Citrullinated Peptide (Anti-CCP) and Anti-Mutated Citrullinated Vimentin (Anti-MCV) Relation with Extra-Articular Manifestations in Rheumatoid Arthritis

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We evaluated the association between anti-cyclic citrullinated peptide antibodies (anti-CCP) and anti-mutated citrullinated vimentin antibodies (anti-MCV) with the presence of extra-articular (ExRA) manifestations in 225 patients with rheumatoid arthritis (RA). Ninety-five patients had ExRA and 130 had no ExRA. There was no association of anti-CCP and anti-MCV levels with the presence of ExRA as total group ($P = 0.40$ and $P = 0.91$, resp.). Making an analysis of individual manifestations, rheumatoid nodules were associated with positivity for rheumatoid factor (RF); ($P = 0.01$), anti-CCP ($P = 0.048$), and anti-MCV ($P = 0.02$). Instead, RF, anti-CCP, or anti-MCV were not associated with SS, chronic anemia, or peripheral neuropathy. Levels of anti-CCP correlated with the score of the Health Assessment Questionnaire-Disability Index (HAQ-Di) ($r = 0.154$, $P = 0.03$), erythrocyte sedimentation rate (ESR); ($r = 0.155$, $P = 0.03$), and RF ($P = 0.254$, $P < 0.001$), whereas anti-MCV titres only correlated with RF ($r = 0.169$, $P = 0.02$). On adjusted analysis, ExRA was associated with longer age ($P = 0.015$), longer disease duration ($P = 0.007$), higher DAS-28 score ($P = 0.002$), and higher HAQ-DI score ($P = 0.007$), but serum levels of anti-CCP and anti-MCV were not associated. These findings show the need to strengthen the evaluation of the pathogenic mechanisms implied in each specific ExRA manifestation.

1. Introduction

Extra-articular manifestations (ExRA) in patients with rheumatoid arthritis (RA) have been observed with at a frequency of 17.8 to 40.9% [1]. These manifestations may involve a multiplicity of organs and are of diverse severity [2]. ExRA are associated with comorbidities, erosions, more aggressive disease, high rate for disability, and premature

mortality [3]. It has been described that patients with ExRA have a 2.5-fold increase in mortality compared with RA without ExRA [2]. Characteristics associated with ExRA include male gender, genetics (HLA-DRB1*04 subtype), positive rheumatoid factor (RF), antinuclear antibodies (ANA), and some environmental factors particularly smoking [4, 5].

Besides of RF and ANA, other autoantibodies have been tested as factors associated with ExRA. Anti-cyclic citrullinated peptide (anti-CCP) antibodies are commonly observed in the serum of RA patients, where the frequency varies between 55% and 69% [6]. Although several studies have evaluated the association of anti-CCP with ExRA, the results have not been always consistent. Turesson et al. in a case-control study found an association of rheumatoid factor (RF) and anti-CCP with the presence of ExRA, although these authors observed a borderline not significant difference in anti-CCP levels in ExRA compared with their controls without ExRA [7]. On the other side, Korkmaz et al. did not observe an association between anti-CCP with ExRA in patients with long disease duration or with early RA [8].

Antibodies against mutated citrullinated vimentin (anti-MCV) have been tested most recently for the diagnosis of RA, showing high sensitivity and specificity for the diagnosis of the disease [9]. Nevertheless, there is a lack of information on studies evaluating the association between anti-MCV with ExRA. To date, to the best of our knowledge, only one study has evaluated the possible association between anti-MCV antibodies with ExRA [10]. These authors did no observe an association between anti-MCV or anti-CCP with ExRA; however, this interesting study did not make explicit the methodology to identify ExRA making it necessary to reevaluate this information [10].

Therefore, because the information about the possible relationship between anti-MCV and ExRA is still insufficient, we designed a study to evaluate whether there is an association of anti-MCV or anti-CCP with ExRA.

2. Material and Methods

2.1. Study Population. We evaluated consecutive patients with RA from an outpatient secondary-care center in Guadalajara, Mexico (Department of Internal Medicine-Rheumatology, Hospital General Regional 110, IMSS). Study participants were included if they were Mexicans mestizo (defined as having at least two generations of ancestors born in Western Mexico) and only one person per family was recruited. To be included, patients with RA had to meet the ACR 1987 criteria for RA to have an established diagnosis and be 18 years old or older. Pregnant or nursing patients, those with other autoimmune disorders such as myasthenia gravis, Hashimoto tiroiditis, or any overlapping syndrome were excluded. Patients with diagnosis of chronic infections including B or C hepatitis, human immunodeficiency virus, tuberculosis, or other chronic infections were also excluded (the assessment for these exclusion criteria was based on the information obtained in a chart review of each patient).

2.2. Study Development. Patients were invited to participate and after signing an informed consent they were assessed by one researcher through a structured interview about epidemiological characteristics (such as age at the time of the study) and disease antecedents; disease duration was defined as the time from the onset of first symptoms of RA until the inclusion in the present study and was assessed through DAS-28 for disease activity, HAQ-DI for functioning, and other clinical measures.

All the included patients were systematically assessed during the evolution of the disease and at the time of the study for presence of eExRA (Table 1), using a structured protocol based on a modification of the criteria described by Turesson et al. [11] to identify patients with ExRA. Briefly, all the patients were systematically assessed by two researchers (both rheumatologists) with a structured interrogatory, physical examination, and chart review; if an ExRA was suspected, this patient was sent for confirmation to an specialist of the organ being involved (for example, ophthalmologist, cardiologist, pulmonologist, dermatologist, and nephrologist). The ExRA assessed included pericarditis (assessed by clinical judgment and confirmed by echocardiography), pleuritis (assessed by clinical judgment and thorax radiographs), Felty's syndrome (based on clinical evidence of splenomegaly confirmed by ultrasound and neutropenia $<1.8 \times 10^9$ described in at least 2 occasions), major cutaneous vasculitis (based on clinical judgment confirmed by biopsy), and neuropathy (based on clinical judgment and positive results poly/mononeuropathy at electromyography). Ocular involvement that was investigated included scleritis, episcleritis, uveitis, or retinal vasculitis (these were diagnosed by ophthalmologists), glomerulonephritis (required being corroborated by nephrologist and renal biopsy if required), vasculitis involving other organs (these were identified by an specialist and if required a biopsy was performed), amyloidosis (based on clinical judgment and positive biopsy if required), keratonconjunctivitis sicca (was assessed in all patients and diagnosed if they had positive Rose-Bengal staining and positive Schirmer's test <5 mm/5 minutes), xerostomia (clinical judgment and abnormal sialometry and if required with minor salivary gland biopsy showing lymphocytic infiltrate), secondary Sjögren's syndrome (SS) (diagnosed if patients met at least two of the following criteria keratonconjunctivitis sicca, xerostomia, positivity for anti-Ro, or anti-La antibodies and lymphocytic infiltrate in minor salivary gland biopsy). Pulmonary involvement included bronchiolitis obliterans or organizing pneumonia (based on clinical judgment by a pulmonologist), whereas pulmonary fibrosis or interstitial lung disease were diagnosed based on clinical judgment by pulmonologist plus restrictive pattern in lung function test and confirmed by positive findings in high-resolution computed tomography of the lung. Cervical myelopathy was assessed by cervical radiographs showing increase in the atlantoaxial distance. Subcutaneous rheumatoid nodules were assessed in the physical examination and the diagnosis based in clinical judgment and biopsy if required. Chronic anemia was diagnosed if a hemoglobin <11 g/dL was observed at least in 3 occasions in the last 6 months before the evaluation and other causes of anemia were excluded. Severe manifestations were considered in presence of pulmonary fibrosis (or other entities

Table 1: Specific extra-articular manifestations evaluated in patients with RA.

Extra-articular manifestation	Criteria
(1) Pericarditis	Clinical judgment and confirmed by echocardiography
(2) Pleuritis	Clinical judgment and thorax radiographs
(3) Felty's syndrome	Clinical evidence confirmed by ultrasound and neutropenia $<1.8 \times 10^9$ described in at least 2 occasions
(4) Major cutaneous vasculitis	Clinical judgment confirmed by biopsy
(5) Neuropathy	Clinical judgment and positive results poly/mononeuropathy at electromyography
(6) Scleritis, epiescleritis, uveitis, or retinal vasculitis	Identified by an specialist and if required a biopsy was performed
(7) Glomerulonephritis	Corroborated by nephrologist and if required a renal biopsy was performed
(8) Vasculitis involving other organs	Identified by an specialist and if required a biopsy was performed
(9) Amyloidosis	Clinical judgment and positive biopsy if required
(10) Keratoncunjunctivitis sicca	Clinical judgment: (a) positive Rose-Bengal staining and (b) positive Schirmer's test <5 mm/5 mn
(11) Xerostomia	Clinical judgment and abnormal sialometry and if suspected minor salivary gland biopsy with lymphocytic infiltrate
(12) Secondary Sjögren's syndrome	Diagnosed by two of the following criteria (a) keratonconjunctivitis sicca, (b) xerostomia, and (c) positivity for anti-Ro or anti-La antibodies
(13) Bronchiolitis obliterans	Clinical judgment by pulmonologist
(14) Organizing Pneumonia	Clinical judgment by pulmonologist
(15) Pulmonary fibrosis	Clinical judgment by pulmonologist plus restrictive pattern in lung function test and confirmed by positive findings in high-resolution computed tomography of the lung
(16) Cervical myelopathy	Clinical judgment and radiograph showing increased in atlantoaxial distance
(17) Subcutaneous rheumatoid nodules	Clinical judgment and biopsy if required
(18) Chronic anemia	Diagnosed if a hemoglobin <11 g/dL was observed in the last 6 months before the evaluation and other causes of anemia were excluded

involving lung), moderate or severe pericarditis/pleuritis, vasculitis involving major organs or cutaneous vasculitis with ulcers or gangrene, mono/polyneuritis multiplex, epiescleritis/scleritis, Felty's syndrome, amyloid deposition, and glomerulonephritis [1].

2.3. Determinations of Anti-MCV and Anti-CCP. At the same day of the clinical evaluation, a venous blood sample was obtained to quantify the titers of anti-CCP and anti-MCV antibodies. This sample was centrifuged and the serum was stored to −20°C until tested. Anti-CCP was detected with ELISA (DIASTAT, Axis-Shield Diagnostics Limited, UK). Positive anti-CCP was defined as a serum concentration ≥5 IU/mL. Sera with anti-CCP levels above the calibration curve were rerun after dilution to obtain actual semiquantitative values for all samples. ELISA kits for detection of anti-MCV (Orgentec Diagnostika GmbH, Mainz, Germany) were used according to manufacturer's instructions with the recommended cut-off value of 20 IU/mL.

2.4. Other Determinations. RF and C-reactive protein (CRP) were quantified in serum by nephelometry using a venous blood sample taken at the same day of the evaluation; the RF was measured on IU/mL and assessed using standard nephelometric assay according to the manufacturer's specifications (Dade Behring, DE). A positive result was defined as a level of >20 UI/mL. CRP was measured on mg/L and assessed using VITROS Chemistry systems CRP Slides, according to the manufacture's specifications (Ortho Clinical Diagnostics, INC.100.Indigo Creek Drive; Rochester NY 14626-5101).

Erythrocyte sedimentation rate (ESR) was determined using Wintrobe technique.

2.5. Statistical Analysis. Quantitative variables were expressed as means and standard deviations and qualitative variables in frequency and percentages. Comparisons in proportions between groups were performed with Chi-square (or Fisher exact test when required) and comparisons in means between groups were performed by Student's t-test for independent samples. A logistic regression analysis of factors associated with ExRA was performed and odds ratios (OR) and 95% confidence intervals (95% CI) were computed for each variable associated with ExRA introduced in the final model.

All analyses were performed two-tailed, and statistical significance was considered when $P \leq 0.05$. All analyses were performed with SPSS software version 8.0.

2.6. Ethics. The study was approved by the Research and Ethics Committee of the Hospital in Guadalajara, Mexico. The study was approved by the Research and Ethics Committee of the Hospital Number of approval R-2010-1303-29. All participants signed a letter of voluntary informed consent.

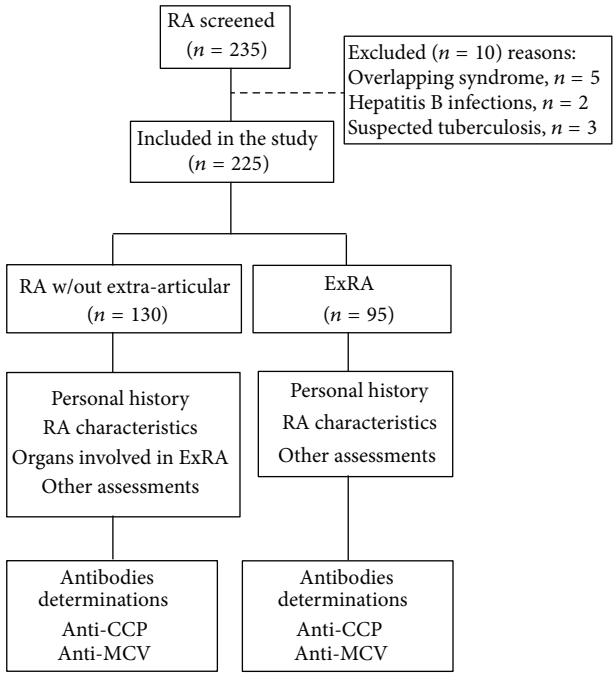

Figure 1: Study flow chart. RA: rheumatoid arthritis; ExRA: extra-articular manifestations; Anti-CCP: Anti-cyclic citrullinated peptide; anti-MCV: antimutated citrullinated vimentin.

The study protocol followed the guidelines of the Helsinki declaration.

3. Results

Figure 1 represents the study flow chart. The total number of patients invited to participate was 235 with RA, of them 10 patients (4.3%) were excluded for the following reasons: five had overlapping syndrome, two had hepatitis B infection, and three were under study for tuberculosis.

Table 2 describes the clinical characteristics of 225 patients with RA included in the study. Most of them were women (92%), the mean age was 52.47 years, and 64 (28.4%) had a history of smoking. Regarding the characteristics of the disease, they had a mean for disease duration for 8.97 years, with a DAS28 score of 5.04 and HAQ-Di score of 0.84, whereas 94 (41.8%) had Steinbrocker radiological stage III or IV in their hands. Of these 225 patients, 95 (42.2%) had ExRA, the most frequently observed was SS in 25.8%, chronic anemia in 14.7%, rheumatoid nodules in 10.2%, and peripheral neuropathy in 5.3%. All the patients included had serological determination of anti-CCP and anti-MCV although only 204 patients had serum determination for RF. The frequencies of positivity for the autoantibodies were RF 66.2%, anti-CCP 68.9%, and anti-MCV 69.7%. The mean levels of RF were 119.11 IU/mL, of anti-CCP were 72.44 IU/mL, and of anti-MCV 156.85 IU/mL. One-hundred and forty patients (62.2%) had positivity for both anti-CCP and anti-MCV antibodies, while 110 patients (53.9%) displayed positivity for anti-CCP, anti-MCV and RF antibodies. Other characteristics of these 225 patients including laboratory variables and their treatments are also shown in this table.

Table 3 compares the clinical characteristics as well as the serological profile of RA patients without ExRA with those with ExRA. Patients with ExRA were older ($P = 0.73$) and had longer disease duration ($P < 0.001$), higher number of tender joints ($P = 0.004$), higher frequency of Steinbrocker radiological stage III or IV in their hands ($P = 0.01$), and higher levels of CRP (P = 0.01). No differences in titers of RF, anti-CCP, or anti-MCV were observed between patients with RA versus ExRA. Regarding the treatments, a higher proportion of patients without ExRA were receiving MTX at the time of the study compared with the group with ExRA ($P = 0.007$) although no statistical significant differences were observed between ExRA and the group without ExRA in MTX dose or anti-TNF agents.

In Table 4, we compare the frequency of specific ExRA of patients with positivity for these autoantibodies versus patients with negativity for these autoantibodies. Frequency of rheumatoid nodules was higher in patients with positive RF (14.1% versus 2.9%, $P = 0.01$), also positive anti-CCP higher frequency of rheumatoid nodules (12.9% versus 4.3%, $P = 0.048$). Similarly, the frequency of rheumatoid nodules was higher in patients with positive anti-MCV as compared with those without these antibodies (13.4% versus 2.9%, $P = 0.02$). Other specific manifestations including SS, chronic anemia, and peripheral neuropathy were not associated with the presence of positivity for these autoantibodies, whereas the number of patients with other specific manifestations such as pulmonary fibrosis, Raynaud syndrome, Felty syndrome, thrombosis, scleritis, or interstitial vasculitis were too low to allow statistical comparisons.

In data not shown in tables, no differences were observed in anti-CCP levels, in patients with SS versus patients without these manifestations (69.92 versus 80.15 IU/mL resp., $P = 0.48$), chronic anemia (72.97 versus 69.51 IU/mL resp., $P = 0.84$), rheumatoid nodules (69.80 versus 95.33 IU/mL resp., $P = 0.18$), or peripheral neuropathy (73.77 versus 45.57 IU/mL resp., $P = 0.17$). Similarly, no differences were observed in serum titers of anti-MCV in SS (160.86 versus 144.84 IU/mL resp., $P = 0.57$), chronic anemia (152.02 versus 184.71 IU/mL resp., $P = 0.42$), rheumatoid nodules (151.59 versus 201.06 IU/mL resp., $P = 0.27$), or peripheral neuropathy (158.92 versus 121.89 IU/mL resp., $P = 0.57$). In a subanalysis, patients with specific manifestations of ExRA were compared regarding to the number of positive autoantibodies that they had. Patients who had rheumatoid nodules had higher frequency of two or more types of autoantibodies compared with patients without nodules (90.9% versus 66.5% resp., $P = 0.019$). On the other side, the number of autoantibodies was not associated with SS ($P = 0.8$), chronic anemia ($P = 0.5$), or peripheral neuropathy ($P = 0.4$). Again total group patients with ExRA were not associated with the number of autoantibodies ($P = 0.6$).

Levels of anti-CCP correlated with the score of the Health Assessment Questionnaire-Disability Index (HAQ-Di) ($r = 0.154$, $P = 0.03$), erythrocyte sedimentation rate (ESR), ($r = 0.155$, $P = 0.03$), and RF ($P = 0.254$, $P < 0.001$),

Table 2: Selected characteristics in patients with rheumatoid arthritis.

Characteristic	RA $n = 225$
Female, *n* (%)	207 (92.0)
Age, years	52.47 ± 10.96
Alcohol consumption*, *n* (%)	28 (12.4)
Smoke exposure, *n* (%)	64 (28.4)
RA characteristics	
Disease duration (years), mean ± SD	8.97 ± 8.32
DAS28, mean ± SD	5.04 ± 1.36
HAQ-Di score (units)	0.84 ± 0.67
Global functional status III-IV, *n* (%)	43 (19.1)
Steinbrocker stage in hands III or IV *n* (%)	94 (41.8)
ExRA, *n* (%)	95 (42.2)
Extra-articular manifestations	
Sjögren's Syndrome, *n* (%)	58 (25.8)
Chronic anemia, *n* (%)	33 (14.7)
Rheumatoid nodules, *n* (%)	23 (10.2)
Peripheral neuropathy, *n* (%)	12 (5.3)
Pulmonary fibrosis, *n* (%)	6 (2.7)
Raynaud's phenomenon, *n* (%)	3 (1.3)
Felty's Syndrome, *n* (%)	2 (0.9)
Thrombosis, *n* (%)	2 (0.9)
Scleritis, *n* (%)	1 (0.4)
Intestinal vasculitis, *n* (%)	1 (0.4)
Laboratory findings	
ESR, mm/h	29.22 ± 11.90
CRP, mg/L	20.85 ± 30.83
Positive RF**, $n = 204$ (%)	135 (66.2)
RF titres, IU/mL	119.11 ± 237.99
Positive anti-CCP	155 (68.9)
Anti-CCP titres, IU/mL	72.44 ± 88.22
Positive anti-MCV	157 (69.7)
Anti-MCV titres, IU/mL	156.85 ± 183.50
(+) anti-CCP and (+) anti-MCV, $n = 225$	140 (62.2)
(+) anti-CCP, (+) anti-MCV and (+) RF, $n = 204$	110 (53.9)
Treatment	
Synthetic DMARDs, *n* (%)	206 (92.6)
MTX users, *n* (%)	180 (80.0)
Anti-TNFα agents, *n* (%)	19 (8.4)
Corticosteroids, *n* (%)	214 (95.1)
Prednisone (current doses), mg/day	5.24 ± 2.06

RA: rheumatoid arthritis; *Alcohol consumption: defined as consume of at least one alcoholic beverage on a daily basis in the last year. DAS28: disease activity score; HAQ-Di: Health Assessment Questionnaire-Disability Index; ExRA: rheumatoid arthritis with extra-articular manifestations; ESR: erythrocyte sedimentation rate; **RF: rheumatoid factor (only assessed in two thousand four patients); CRP: C-Reactive Protein; anti-CCP: anti-cyclic citrullinated peptide antibodies; anti-MCV: anti-mutated citrullinated vimentin, DMARDs: disease-modifying antirheumatic drugs.
Qualitative variables are expressed as frequencies (%); quantitative variables are expressed as means and standard deviation.

Table 3: Comparisons of characteristics between patients with ExRA and without extra-articular manifestations (RA w/out).

Clinical characteristics	RA w/out $n = 130$	ExRA $n = 95$	P
Female, *n* (%)	116 (89.2)	91 (95.8)	0.73
Age, years	50.75 ± 10.92	54.82 ± 10.62	0.006
Alcohol consumption, *n* (%)	21 (16.2)	7 (7.4)	0.49
Smoke exposure, *n* (%)	30 (23.1)	34 (35.8)	0.04
Disease duration, years	7.04 ± 6.57	11.60 ± 9.69	<0.001
DAS28	5.03 ± 1.34	5.05 ± 1.39	0.91
Tender joint count	4.28 ± 4.91	7.40 ± 7.18	0.004
Swollen joint count	6.42 ± 6.89	7.47 ± 8.08	0.43
VAS global	42.71 ± 26.48	42.80 ± 28.70	0.16
HAQ-Di score (units)	0.89 ± 0.66	0.77 ± 0.69	0.18
Global functioning status III-IV, *n* (%)	23 (17.7)	20 (21)	0.53
Radiological Stage III or IV in hands, *n* (%)	45 (34.7)	49 (51.6)	0.01
ESR, mm/h	29.40 ± 11.56	28.97 ± 12.40	0.79
CRP, mg/L	15.43 ± 19.84	27.31 ± 39.36	0.01
RF, IU/mL	119.07 ± 252.69	119.16 ± 220.03	0.99
Anti-CCP, IU/mL	67.99 ± 74.14	78.62 ± 104.86	0.40
Anti-MCV, IU/mL	155.60 ± 179.03	158.58 ± 190.44	0.91
MTX users, *n* (%)	112 (86.2)	68 (71.6)	0.007
MTX dose, mean ± SD	7.33 ± 4.14	6.37 ± 5.16	0.12
Anti-TNF agents users, *n* (%)	9 (6.9)	10 (10.5)	0.34

RA w/out: rheumatoid arthritis (RA) without extra-articular manifestations; ExRA: RA with extra-articular manifestations; DAS28: disease activity score; HAQ-Di: Health Assessment Questionnaire-Disability Index; ESR: erythrocyte sedimentation rate; RF: rheumatoid factor; CRP, C-Reactive Protein; anti-CCP: anti-cyclic citrullinated peptide antibodies; anti-MCV: anti-mutated citrullinated vimentin, DMARDs: disease-modifying antirheumatic drugs. Qualitative variables are expressed as frequencies (%); quantitative variables are expressed as mean and standard deviation. Comparisons between proportions were compared with Chi-square or Fisher exact test (when required). Comparisons between means were evaluated with Student's *t*-test for independent samples.

Table 4: Comparisons of frequencies of ExRA and specific extra-articular manifestations according to the findings of positive or negative anti-CCP or anti-MCV.

Characteristics	(−) RF $n = 69$	(+) RF $n = 135$	P	(−) anti-CCP $n = 70$	(+) anti-CCP $n = 155$	P	(−) anti-MCV $n = 68$	(+) anti-MCV $n = 157$	P
Presence of ExRA, *n* (%)	30 (43.5)	57 (42.2)	0.86	27 (38.6)	68 (43.9)	0.46	26 (38.2)	69 (43.9)	0.43
Specific manifestations									
Sjögren syndrome, *n* (%)	23 (33.3)	29 (21.5)	0.07	18 (25.7)	40 (25.8)	1.00	16 (23.5)	42 (26.8)	0.61
Chronic anemia, *n* (%)	9 (13.0)	23 (17.0)	0.46	7 (10.0)	26 (16.8)	0.18	9 (13.2)	24 (15.3)	0.69
Rheumatoid nodules, *n* (%)	2 (2.9)	19 (14.1)	0.01	3 (4.3)	20 (12.9)	0.048	2 (2.9)	21 (13.4)	0.02
P. neuropathy, *n* (%)	6 (8.7)	5 (3.7)	0.19	5 (7.1)	7 (4.5)	0.52	4 (5.9)	8 (5.1)	0.76

The cut-off value to be considered anti-CCP as positive was ≥5 IU/mL, for anti-MCV, the levels considered as positive were ≥20 IU/mL. RA: rheumatoid arthritis; ExRA: extra-articular manifestations; anti-CCP: anti-cyclic citrullinated peptide antibodies; anti-MCV: anti-mutated citrullinated vimentin antibodies, P. neuropathy (peripheral neuropathy). Qualitative variables are expressed as frequencies (%). Comparisons between proportions were compared with Fisher exact test.

whereas anti-MCV titres only correlated with RF ($r = 0.169$, $P = 0.02$).

Table 5 shows a comparison of clinical characteristics between patients who presented a specific ExRA versus those who did not. Patients with SS had lower age ($P = 0.001$) and lower disease duration ($P = 0.005$), also had a higher proportion of patients diagnosed before year 2000 ($P = 0.009$), higher prevalence of Chloroquine treatment ($P = 0.008$), and lower prevalence of MTX treatment ($P = 0.04$). Instead, patients with anemia had a lower DAS28 ($P = 0.005$) and lower CRP levels ($P = 0.015$). In patients with rheumatoid nodules lower disease duration ($P = 0.001$),

Table 5: Comparison in characteristics between specific ExRA manifestations and w/out these manifestations.

Clinical characteristics	Sjögren's syndrome		P	Chronic anemia		P	Rheumatoid nodules		P	Peripheral neuropathy		P
	Yes	No		Yes	No		Yes	No		Yes	No	
	$n = 58$	$n = 167$		$n = 33$	$n = 192$		$n = 23$	$n = 202$		$n = 12$	$n = 213$	
Female, n (%)	56 (96.6)	151 (90.4)	0.17	32 (97.0)	175 (91.1)	0.48	22 (95.7)	185 (91.6)	0.70	12 (100)	195 (91.5)	0.29
Age, years	51.0 ± 11	56.7 ± 9.1	0.001	52.9 ± 11	50.0 ± 12	0.17	52.1 ± 11	55.1 ± 12	0.22	52.1 ± 11	58.0 ± 7.8	0.07
Disease duration, years	7.9 ± 7.5	11.9 ± 9.7	0.005	9.0 ± 8.4	8.5 ± 7.6	0.74	8.1 ± 7.7	16.2 ± 9.7	0.001	8.8 ± 8.3	12.0 ± 7.4	0.19
Year of diagnosis before 2000	20 (34.5)	30 (18.0)	0.009	6 (18.2)	44 (22.9)	0.55	10 (43.5)	40 (19.8)	0.010	4 (33.3)	46 (21.6)	0.31
DAS28	5.1 ± 1.3	4.9 ± 1.3	0.29	4.9 ± 1.3	5.6 ± 1.2	0.005	5.03 ± 1.3	5.11 ± 1.3	0.79	5.1 ± 1.3	4.6 ± 1.5	0.21
HAQ-Di score (units)	0.88 ± 0.67	0.72 ± 0.66	0.11	0.9 ± 0.7	0.8 ± 0.7	0.81	0.9 ± 0.7	0.7 ± 0.6	0.29	0.9 ± 0.7	0.7 ± 0.6	0.49
Radiological III-IV hands, n (%)	28 (48.3)	66 (39.5)	0.24	17 (51.5)	77 (40.1)	0.22	16 (69.6)	78 (38.6)	0.004	7 (58.3)	87 (40.8)	0.23
ESR, mm/h	30.0 ± 11.9	26.8 ± 11.6	0.08	28.9 ± 12	31.1 ± 14	0.33	29.0 ± 11.9	30.8 ± 11.7	0.51	29.5 ± 12	23.5 ± 10	0.09
CRP, mg/L	21.5 ± 32.1	19.1 ± 27.0	0.62	16.8 ± 22.2	40.7 ± 52.5	0.015	19.6 ± 28.5	31.1 ± 45.1	0.26	21.6 ± 32	9.2 ± 9	0.19
(+) RF*, n (%)	29 (55.8)	106 (69.7)	0.07	23 (71.9)	112 (65.1)	0.46	19 (90.5)	116 (63.4)	0.01	5 (45.5)	130 (67.4)	0.19
(+) Anti-CCP, n (%)	40 (69.0)	115 (68.9)	0.99	26 (78.8)	129 (67.2)	0.18	20 (87.0)	135 (66.8)	0.048	7 (58.3)	148 (69.5)	0.52
(+) Anti-MCV, n (%)	42 (72.4)	115 (68.9)	0.61	24 (72.7)	133 (69.3)	0.69	21 (91.3)	136 (67.3)	0.02	8 (66.7)	149 (70.0)	0.76
Chloroquine users, n (%)	14 (24.1)	17 (10.2)	0.008	4 (12.1)	27 (14.1)	1.00	4 (17.4)	27 (13.4)	0.53	1 (8.3)	30 (14.1)	1.00
MTX users, n (%)	41 (70.7)	139 (83.2)	0.04	26 (78.8)	154 (80.2)	0.85	12 (52.2)	168 (83.2)	0.001	8 (66.7)	172 (80.8)	0.26
MTX doses, mean ± SD	7.2 ± 4.4	6.2 ± 5.0	0.15	6.8 ± 4.4	7.6 ± 5.5	0.46	7.0 ± 4.4	5.7 ± 6.0	0.32	7.0 ± 4.6	4.8 ± 4.4	0.10
Azathioprine users, n (%)	6 (10.3)	13 (7.8)	0.55	5 (15.2)	14 (7.3)	0.17	9 (39.1)	10 (5.0)	<0.001	2 (16.7)	17 (8.0)	0.27
Anti-TNF agents, users, n (%)	6 (10.3)	13 (7.8)	0.59	2 (6.1)	17 (8.9)	1.00	4 (17.4)	15 (7.4)	0.11	0 (0.0)	19 (8.9)	0.61

RA: rheumatoid arthritis; DAS28: disease activity score; HAQ-Di: Health Assessment Questionnaire-Disability Index; ExRA: rheumatoid arthritis with extra-articular manifestations; ESR: erythrocyte sedimentation rate; CRP: C-Reactive Protein; *RF: rheumatoid factor (only assessed in two thousand four patients); anti-CCP: anti-cyclic citrullinated peptide antibodies; anti-MCV: anti-mutated citrullinated vimentin, DMARDs: disease-modifying antirheumatic drugs. Qualitative variables are expressed as frequencies (%); quantitative variables are expressed as means and standard deviation. Comparisons between proportions were made with Chi-square (or Fisher exact test if required). Comparisons between means were made using Student's t-test.

higher proportion of patients diagnosed before year 2000 ($P = 0.010$), higher proportion of Steinbrocker's score III or IV ($P = 0.004$), higher proportion of +RF ($P = 0.01$), higher frequency of +anti-CCP ($P = 0.048$), anti-MCV ($P = 0.02$), lower frequency of MTX utilization ($P = 0.001$), higher proportion of azathioprine users ($P < 0.001$) were observed.

Table 6 shows the results of the multivariate regression analysis of variables associated with ExRA presence. Using the forward stepwise method, variables associated with ExRA were age ($P = 0.015$), disease duration ($P = 0.007$), DAS-28 ($P = 0.002$), and HAQ-DI ($P = 0.007$). No significant associations were identified between presence of ExRA with anti-CCP and anti-MCV levels.

4. Discussion

Our results showed that 42% of our patients had ExRA; the variables associated with this involvement in the adjusted analysis are older age, longer disease duration, DAS-28 score, and HAQ-DI score, whereas no association was observed between anti-CCP or anti-MCV antibodies and ExRA as general group. For specific ExRA, there was a weak association between the presence of rheumatoid nodules and positivity for RF, anti-CCP, or anti-MCV antibodies but no relationship was found between these autoantibodies and SS, chronic anemia, or peripheral neuropathy.

The frequency of ExRA in RA observed in our study is similar to that described by Turesson et al. [12], where they observed that 40.6% of their patients with RA had ExRA. A review of 12 studies evaluating ExRA in RA revealed a variation of 17.8% to 40.9%; this frequency varies depending on the study designs, methodology, and definitions used for the detection of ExRA as well as characteristics of the center from where the patients were procured [1]. The most frequent ExRA observed in our study were SS, chronic anemia, and rheumatoid nodules. Among these different studies, the frequency of SS and Sicca syndrome vary from 7.3% to 19.6%, whereas the frequency of chronic anemia varies between 30% and 70% and rheumatoid nodules have been reported from 2.1% to 38.2% [1, 13].

Because the main objective of the study was to identify the possible association of anti-CCP or anti-MCV autoantibodies with ExRA, we examined primarily all the patients that had one or more ExRA and subsequently, those patients were with a specific ExRA. We did not observe an association between the presence of ExRA as total group and positivity for anti-CCP. Similar results were reported by Sghiri et al. who did not observe association between ExRA and anti-CCP antibodies [10]. Our data differ from observations made by Salinas et al. [14] who described a significant association between anti-CCP and ExRA. On the other side, Turesson et al. observed an association between anti-CCP and ExRA but only in those patients with severe ExRA, whereas this association does not persisted when ExRA was evaluated in general [7]. Unfortunately, a limitation in our study was that the number of patients with severe ExRA was too small to allow statistical comparisons. Regarding the SS, we did not observe an association between SS and positivity for anti-CCP or anti-MCV antibodies. While some groups designate the SS associated to RA as a "secondary" SS, this syndrome could be considered as an overlap disease more than an ExRA [15]. Barcelos et al. identified that patients with secondary SS had higher frequency of anti-CCP antibodies [16] although their sample was small and therefore is limited in its appropriateness to generalize their results. We have recently informed the results of a case-control study performed obtained from a different population where we observed a significant association between positivity for anti-CCP antibodies and the presence of interstitial lung disease associated to RA (ILD-RA) [17]. Nevertheless, in this present study, we were limited to evaluate this association because the low prevalence observed of ILD-RA.

We did not observe an association between anti-MCV and ExRA as total group. Our data are similar to those described by Sghiri et al. [10]. Although, as described above, we have found a weak but significant association between positivity for this autoantibody and rheumatoid nodules, finding that requires to be reproduced by other studies. An association was observed between the nonutilization of MTX and ExRA, although this association was not observed with dose or anti-TNF agents. Mikuls et al. identified that during the course of treatment, around a half of their patients experienced a decrease in RF or anti-CCP levels [18]. These data are in concordance with our findings related to lower frequency of ExRA in MTX users. On the other side Nicaise Roland et al. have described that patients with RA treated with infliximab may have a decrease in anti-CCP and anti-MCV levels with the therapy [19]. We did not identified statistical differences on the frequency of ExRA in users of anti-TNF agents, nevertheless, a clear limitation for our analysis is the small proportion of patients that were treated with these agents.

Our study has several limitations, first because our study was designed to include consecutive patients with RA tested for presence of ExRA and although we evaluated 225 patients, we were unable to identify a significant number of severe ExRA, therefore, we did not test associations in these subgroups. Nevertheless, we identified a significant number of patients with nonsevere ExRA and found no associations except for rheumatoid nodules. Another limitation inherent to this cross-sectional design is that we only tested for anti-CCP and anti-MCV antibodies in one occasion, being necessary in further studies to test these auto-antibodies several times to identify variations in their titers. Hence, further longitudinal studies are required to test if patients with RA with higher titers for these autoantibodies will develop a higher rate of ExRA in the long-run. Another limitation of our study is the long disease duration observed in some of our patients, in this case is expected that the age of our patients at the time of the study is closely related with RA duration. Therefore we observed in the multivariate analysis that disease duration is relevant confounder in the final model.

Relevantly, in the best of our knowledge this is the first study performed in Mexican patients that test the association between anti-CCP or anti-MCV anti-bodies with ExRA, and

Table 6: Multivariate logistic regression testing for variables associated with ExRA.

Criterion predictor	Method Enter			Method forward stepwise		
	OR	95% CI	*P*	OR	95% CI	*P*
Age, years	1.08	1.02–1.13	0.002	1.05	1.01–1.10	0.015
Disease duration, years	1.13	1.04–1.23	0.002	1.09	1.02–1.16	0.007
Smoke exposure	0.70	0.25–2.00	0.41	Not in the model	—	—
DAS-28	2.24	1.42–3.52	<0.001	1.86	1.27–2.73	0.002
Functional Impairment (HAQ-Di)	5.62	1.78–17.75	0.003	4.46	1.51–13.14	0.007
CRP mg/L	1.02	1.00–1.05	0.025	Not in the model	—	—
ESR mm/hr	0.95	0.90–0.99	0.02	Not in the model	—	—
RF IU/mL	1.27	0.45–3.54	0.64	Not in the model	—	—
Anti-CCP IU/mL	1.00	0.99–1.01	0.84	Not in the model	—	—
Anti-MCV IU/mL	1.00	0.99–1.002	0.63	Not in the model	—	—
Anti-TNF agents	1.63	0.36–7.50	0.53	Not in the model	—	—

DAS28: disease activity score 28-joints assessed; HAQ-Di: Health Assessment Questionnaire-Disability Index; MTX: methotrexate; Anti-CCP: anti-cyclic citrullinated peptide antibodies, OR: odds ratio, 95% CI, 95% confidential intervals.

also is one of the few studies where patients are obtained from a secondary-care center; this last characteristic constitutes an advantage compared with studies performed in tertiary-care centers where ExRA is expected be more severe and are more likely to have referral bias.

In conclusion, we identified that 42% of our patients with RA have extra-articular manifestations. Variables associated with ExRA were age, disease duration, DAS-28 score, and HAQ-DI score. This study did not observe an association between ExRA as total group and the positivity or titers of anti-CCP or anti-MCV antibodies. Although a weak association was observed between positivity for these autoantibodies and rheumatoid nodules. Further studies with more severe ExRA should be performed to identify if these autoantibodies are associated with these specific manifestations.

Conflict of Interests

All authors declare that there is no conflict of interests regarding the publication of this paper.

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Review Article

Acquired Hemophilia A: A Frequently Overlooked Autoimmune Hemorrhagic Disorder

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Acquired hemophilia A (AHA) is a rare hemorrhagic disease in which autoantibodies against coagulation factor VIII- (FVIII-) neutralizing antibodies (inhibitors) impair the intrinsic coagulation system. As the inhibitors developed in AHA are autoantibodies, the disease may have an autoimmune cause and is often associated with autoimmune disease. Although acute hemorrhage associated with AHA may be fatal and is costly to treat, AHA is often unrecognized or misdiagnosed. AHA should thus be considered in the differential diagnosis particularly in postpartum women and the elderly with bleeding tendency or prolonged activated partial thromboplastin time. Cross-mixing tests and measurement of FVIII-binding antibodies are useful to confirm AHA diagnosis. For treatment of acute hemorrhage, hemostatic therapy with bypassing agents should be provided. Unlike in congenital hemophilia A with inhibitors, in which immune tolerance induction therapy using repetitive infusions of high-dose FVIII concentrates is effective for inhibitor eradication, immune tolerance induction therapy has shown poor efficacy in treating AHA. Immunosuppressive treatment should thus be initiated to eradicate inhibitors as soon as the diagnosis of AHA is confirmed.

1. Introduction

During the course of treatment for autoimmune disease, patients with no history of bleeding sometimes suddenly present with severe ecchymoses or muscle hematoma. In such cases, acquired coagulation factor deficiencies, including acquired hemophilia A (AHA), should be considered in the differential diagnosis of the cause of bleeding [1]. As a rare hemorrhagic disorder but the most frequently acquired coagulation factor deficiency, AHA is caused by the development of antibodies, referred to as "inhibitors," against coagulation factor VIII (FVIII), which neutralize FVIII activity. Although AHA has previously been reported to have an incidence of 0.2 to 1.0 cases per million population per year [2], a recent report describes a progressively increasing incidence of 2 cases per million population per year [3], likely resulting from greater awareness of the disorder. In contrast to the incidence of congenital hemophilia A, a recessive X-linked genetic disorder, the incidence of AHA has not been found to differ significantly between men and women. AHA has a biphasic age distribution, exhibiting a small peak from age 20 to 30 years and a larger peak at age 60 years and older [4, 5]. The majority of patients who present with AHA between ages 20 and 30 years are female, as the disease in this age group is associated with pregnancy (i.e., the development of postpartum inhibitors) and autoimmune disorders. While it was previously thought that the majority of patients who present with AHA at age 60 years and older are male [4, 6], recent studies have revealed no significant difference in the sex ratio of elderly patients [7].

While AHA has a high mortality rate, estimated at up to 33%, it has decreased in tandem with the advancement of therapeutic interventions since the 1980s [8]. AHA occurs relatively less frequently but develops suddenly and occasionally presents with life-threatening bleeding. Furthermore, the management of AHA remains difficult and the costs of treatment are often immense. Although AHA is thus clinically and economically an important disorder, it is often unrecognized or misdiagnosed as other acquired hemorrhagic disorders, such as disseminated intravascular coagulation (DIC) and

acquired inhibitors against von Willebrand factor (acquired von Willebrand syndrome [9]) and factor XIII (acquired factor XIII deficiency [10]).

In contrast to the FVIII-neutralizing inhibitors that develop in congenital hemophilia A after FVIII-replacement therapy, which are alloantibodies, the FVIII-neutralizing inhibitors that develop in AHA are autoantibodies. It is well known that approximately 50% of patients with AHA have or have had immune system disorders, such as autoimmune diseases and lymphoproliferative disorders. This fact, as well as knowledge that autoantibodies play a central role in AHA pathogenesis, indicates that modulation of the immune system or the autoimmune mechanism that generates autoantibodies is involved in AHA.

2. Clinical Manifestations

AHA patients often present with severe and massive bleeding, which is responsible for their relatively high mortality rate. The most commonly affected organ is the skin, especially at the site of injection or contusion, which often manifests severe ecchymoses. Subsequently, intramuscular and gastrointestinal/intra-abdominal bleedings are often involved. It is notable that hemarthroses most commonly appear in congenital hemophilia A but seldom occur or cause joint damage in AHA [11, 12]. AHA is also associated with postdelivery or postoperative bleeding. Although relatively uncommon, intra-abdominal or intracerebral hemorrhage in AHA patients often leads to life-threatening bleeding. Persistent bleeding after surgical procedures, such as intramuscular injection, catheter insertion, and tracheotomy for treatment of underlying or incidentally coexisting diseases, may be the earliest symptom of AHA. Occasionally, AHA is suspected despite the absence of hemorrhagic manifestations by review of the preoperative examination results, especially in patients with low-titer inhibitors. A notable prognostic consideration is that, unlike in congenital hemophilia A, inhibitor titer in AHA does not indicate the severity or frequency of bleeding.

3. Characteristics of AHA Inhibitors

3.1. FVIII. FVIII is a cofactor for activated factor IX (FIXa) that forms the Xase (tenase) complex in the presence of Ca^{2+} and phospholipids and is essential for the intrinsic coagulation system responsible for blood clotting; therefore, FVIII deficiency causes dysfunction of the intrinsic system and reduces thrombin generation, resulting in a bleeding disorder. FVIII is mainly synthesized in the liver as a 2,351 amino acid and 330-kDa single-chain precursor glycoprotein with a functional domain structure (A1-A2-B-A3-C1-C2) (Figure 1) [13]. After proteolytic processing, circulating mature FVIII protein is composed of a heterodimer of a heavy (A1-A2) and a light (A3-C1-C2) chain. This chain is noncovalently bound to von Willebrand factor (VWF), which protects the FVIII from inactivation. VWF has a molecular weight of 226 kDa and a multimeric structure consisting of subunits of large molecular weight (>20,000 kDa).

3.2. Characteristics of Inhibitors. The majority of FVIII inhibitors observed in AHA, which are polyclonal autoantibodies, and in congenital hemophilia A, which are polyclonal alloantibodies, bind to the A2 (454–509), A3 (1804–1819), or C2 domains (2181–2243) [14–17]. While anti-C2 antibodies interfere with the binding of FVIII to phospholipids and VWF, A2 and A3 inhibitors block the binding of FVIII to factor X (FX) and FIXa, respectively, and obstruct the formation of the Xase complex.

Previous studies of CD4 T-cell subsets (Th1, Th2, and Th3) specific for FVIII revealed that alloantibodies in congenital hemophilia A consist of Th1-dependent immunoglobulin (Ig) G1 and IgG2 and Th2-dependent IgG4. However, AHA autoantibodies are often IgG4 autoantibodies and less frequently IgG1 and IgG2 autoantibodies. Further, FVIII-neutralizing activity is correlated with the presence of IgG4 autoantibodies [3, 18, 19]. As IgG4 antibodies form non-precipitating immune complexes and are not complement-fixing autoantibodies, they do not cause the severe organ damage often seen in hemophilia B patients, in whom allergic reactions to FIX concentrates are associated with the specific IgG1 subclass of alloantibodies against FIX [20].

Most alloantibodies developed in congenital hemophilia A patients undergoing FVIII replacement therapy, which are classified as type I inhibitors of first-order kinetics, inactivate FVIII at a rate linearly correlated with their concentration and are able to completely inhibit FVIII activity at high concentrations. In contrast to the kinetics of the interaction between FVIII and the inhibitors in congenital hemophilia A, the kinetics of the interaction in AHA display a non-linear inhibitory profile. Specifically, these type II inhibitors show a rapid initial inactivation phase followed by a slower equilibrium phase during which some residual FVIII activity (FVIII:C) is detectable even after incubation at maximum concentrations of inhibitors for a sufficient period (Figure 2). However, AHA patients with identifiable FVIII:C manifest far more severe hemorrhage than congenital hemophiliacs with comparable levels of FVIII:C. Moreover, addition of excessive FVIII concentrates fails to neutralize the inhibitory activity of type II inhibitors in vitro, making management of AHA difficult in the clinical setting and high-dose replacement therapy with FVIII concentrates unsuccessful in AHA patients with high-titer inhibitors.

In a study of the physiological activities of AHA inhibitors, Lacroix-Desmazes et al. identified a subset of inhibitors in congenital hemophilia A that hydrolyze FVIII, resulting in FVIII inactivation [21, 22]. On the basis of their findings, Lacroix-Desmazes et al. advocated a unique conception of the inhibitory mechanism in AHA that has been supported by further research of the proteolytic activity of IgG isolated from patients with AHA, demonstrating the presence of autoimmune FVIII-hydrolyzing IgG. On the basis of the observation that the extent of the FVIII hydrolytic activity of acquired inhibitors exhibits a correlation with inhibitory titers of the inhibitors, IgG-mediated FVIII hydrolysis has been hypothesized to participate in FVIII inactivation in AHA [23]. This hypothesis is supported by the results of a comparison study of the properties of the proteolytic inhibitors in congenital hemophilia

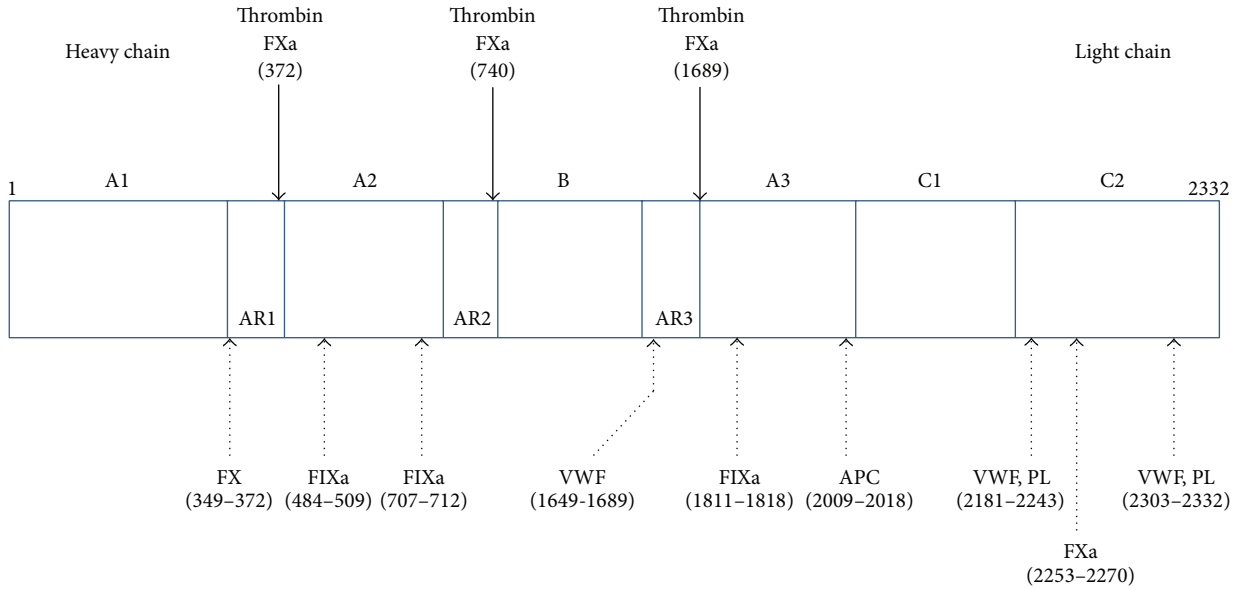

Figure 1: Structure of the coagulation factor VIII (FVIII) molecule. The numbers indicate amino acid positions. Plasma FVIII is a heterodimer composed of a heavy chain (domains A1, A2, and B) and a light chain (domains A3, C1, and C2). Noncovalent binding of FVIII with von Willebrand factor (VWF) protects circulating FVIII from being inactivated by activated protein C. The binding sites of VWF, phospholipids (PL), and other coagulation factors (activated factor IX [FIXa], factor X [FX], and activated FX [FXa]) are also indicated. FVIII is cleaved and activated by thrombin and FXa at residues 372 and 740 within the heavy chain and at residue 1689 within the light chain. Inhibitors impair FVIII activation by interfering with thrombin-catalyzed cleavage or FVIII interactions with VWF, FIXa, FX, and PL. AR: acidic region.

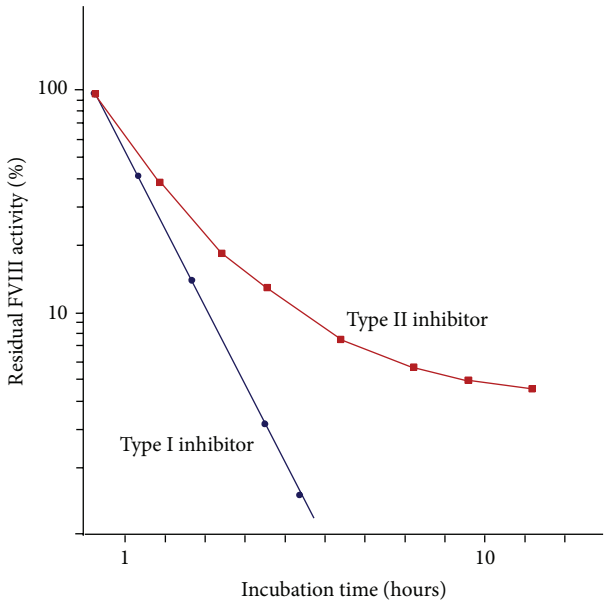

Figure 2: Kinetics of type I and type II inhibitors.

A and AHA, which, using proline-phenylalanine-arginine-methylcoumarinamide (PFR-MCA), a synthetic substrate for FVIII-hydrolyzing autoantibodies, revealed that the rate of FVIII hydrolysis differs significantly between hemophilia A and AHA patients. While the results of the PFR-MCA and Bethesda assay revealed a correlation between hydrolytic activity and inhibitor titer in acquired inhibitors, alloantibodies in congenital hemophilia A exhibit little correlation. These findings suggest that populations of proteolytic inhibitors in AHA patients differ from those in congenital hemophilia A patients with inhibitors [24]. In addition, some AHA autoantibodies can augment FIX activity by FIX proteolysis in the absence of FVIII-proteolytic activity. On the basis of these findings, it has been hypothesized that the FIX-potentiating action of autoantibodies may partially compensate for the inhibition of FVIII, resulting in restoration of thrombin generation [25].

4. Underlying Conditions in AHA

In approximately 50% of AHA patients, especially elderly patients, autoantibody development against factor VIII is idiopathic [2, 26, 27], indicating that the acquired inhibitors develop via an autoimmune mechanism. The underlying conditions shown in Table 1 are observed in the remaining 50% of patients.

4.1. Autoimmune Diseases. AHA is often associated with autoimmune diseases, including rheumatoid arthritis, systemic lupus erythematosus, myasthenia gravis, multiple sclerosis, thyroid dysfunction, and autoimmune hemolytic anemia. Observation of an association between AHA and inflammatory bowel disease, pemphigus, and graft versus host disease (GVHD) has been also reported, indicating that AHA has an autoimmune origin. In fact, up to 20% of all AHA patients present with autoimmune disorders [28]. As

Table 1: Conditions associated with acquired hemophilia A.

Idiopathic	Malignancy
Autoimmune diseases	Squamous cell cancer
Rheumatoid arthritis	Lymphoproliferative diseases
Systemic lupus erythematosus	Chronic lymphocytic leukemia
Myasthenia gravis	non-Hodgkin lymphoma
Multiple sclerosis	Multiple myeloma
Thyroid dysfunction	Medical agents
Autoimmune hemolytic anemia	Antibiotics
Inflammatory bowel diseases	Penicillins
Pemphigus	Sulfonamides
Graft versus host disease	Chloramphenicol
Pregnancy	Anticonvulsants (phenytoin)
	Antihypertensive (methyldopa)
	Bacillus Calmette-Guérin vaccination

FVIII inhibitor titers in patients with autoimmune disorders are often high and less frequently resolve spontaneously compared to those associated with pregnancy, the former require aggressive treatment for bleeding management and inhibitor eradication consisting of both hemostatic therapy using bypassing agents and immunosuppressive therapy.

4.2. Pregnancy. AHA is associated with pregnancy in approximately 10% of cases [8]. Although hemorrhagic symptoms commonly present between 1 and 4 months after parturition, they may occur over a year after delivery [29, 30]. While the hemorrhagic potential is often low and the inhibitors often spontaneously disappear in almost all patients with low titers of inhibitors [29], it may be difficult to achieve inhibitor eradication in patients with high titers (≥5 BU/mL), even with aggressive immunosuppressive therapy. An important consideration is that carrying a fetus might pose the risk of fatal bleeding, as it poses the risk of diaplacental transition of inhibitor IgG from pregnant AHA patients [30]. When inhibitor eradication in patients with postpartum inhibitors is unsuccessful, other commonly associated conditions, especially autoimmune disorders, should be suspected.

4.3. Malignancy. Underlying malignancy in either solid or nonsolid form presents in approximately 10% of AHA patients and commonly develops in elderly patients. An important consideration is that, as the incidence of both solid tumor as well as AHA increases with aging, inhibitors might be detected coincidentally in patients with solid tumor. Patients with lymphoproliferative diseases complicating AHA, which include chronic lymphocytic leukemia, non-Hodgkin lymphoma, and multiple myeloma [8], with altered immune status often have coexisting autoimmune diseases, one of which may be AHA. As anticarcinogenic agents induce cell damage and/or modulate immunological reactions through danger signals [31, 32], patients with malignancies might be predisposed to autoimmune phenomena and increased risk of developing inhibitors. Although it remains unclear whether autoantibody development derives from the tumor itself, the observation that cancer antigens share immunological cross-reactivity with FVIII has not been reported to date.

4.4. Medical Agents. Reactions associated with drug hypersensitivity have been implicated in the onset of AHA. Suspected medications include antibiotics (penicillin, sulfonamides, and chloramphenicol), anticonvulsants (phenytoin), antihypertensive agents (methyldopa), and bacillus Calmette-Guérin vaccination [2]. As high titers of inhibitors resulting from drug reactions and allergies disappear after termination of the responsible drug [29], specific therapy to eradicate FVIII autoantibodies may not be provided to patients who experience drug hypersensitivity. It is notable that administration of interferon for hepatitis C virus infection, which, by directly acting on the immune system, results in malfunctioning of the immune response, is associated with AHA [33].

5. Molecular Biological Mechanisms

Anti-FVIII autoantibodies are developed in the context of dysfunction of immune system, as discussed above. Knowledge of the detailed molecular biological mechanism of inhibitor generation has accumulated gradually over the past decades.

5.1. CTLA-4. Variants of the polymorphic cytotoxic T lymphocyte antigen-4 (CTLA-4) gene, which is found on the surface of activated and regulatory T-cells, have been associated with autoimmune diseases [34]. The extracellular domain of CTLA-4 is similar to the domain of the CD28 that is a component of the costimulatory CD28/B7 receptor/ligand system and competes against CD28 ligands, such as CD80 and CD86, on the surface of dendritic cells. Stimulation of the CD28 receptor on T-cells emits a costimulatory signal for T-cell proliferation and activation. In contrast, CTLA-4 may inhibit T-cell activation by restricting the ability of B7 to interact with CD28 [35]. In regulatory T-cells, CTLA-4 is constitutively expressed at a steady state through transcriptional enhancement by Foxp3. Tight binding of CTLA-4 molecules

on regulatory T-cells to costimulatory ligand B7 on antigen-presenting cells strips and destroys B7 molecules. As antigen-presenting cells without B7 ligands cannot deliver additional Signal 2 (i.e., engage in costimulation), the binding of CTLA-4 and costimulatory ligand B7 terminates activation or differentiation of naïve T-cells to effector T-cells. Thus, CTLA-4 acts as a receptor that downregulates the immune system. The results of several studies, including those of a recent study that observed a single nucleotide polymorphism of the CTLA-4 gene (+49 A/G allele) at a significantly higher frequency in AHA patients compared with controls [36], indicate that CTLA-4 variants (CTLA-4 single nucleotide polymorphisms) might also be involved in the pathogenesis of AHA as well as that other genetic/environmental factors might contribute to the onset of AHA.

5.2. BAFF. Recently, B-cell activating factor belonging to the tumor necrosis factor family (BAFF), also referred to as BlyS, has been found to regulate the immune system. Known to be involved in the survival and maturation of B-cells [37], BAFF binds to tumor necrosis factor-related receptors, such as B-cell-maturation antigen (BCMA), transmembrane-activator and calcium-modulator and cyclophilin-ligand interactor (TACI), and B-cell activating factor receptor (BAFF-R) [38]. This BAFF-mediated ligand-receptor interaction forms a complex network that plays a critical role in the induction and regulation of humoral immunity. Previous mouse studies demonstrated that constitutive BAFF overexpression leads to survival of autoreactive B-cells [39], which in turn induces breakdown of peripheral tolerance. In this setting, autoimmune disorders develop through anomalous B-cell activation with spontaneous production of multiple autoantibodies and polyclonal hypergammaglobulinemia. In humans, elevated BAFF levels are associated with several B-cell-mediated autoimmune diseases with hypergammaglobulinemia [40–42].

In a previous study, we found BAFF levels to be significantly higher in congenital hemophilia A patients with inhibitors compared to healthy controls or hemophilia A patients without inhibitors [43]. These results suggest that elevated BAFF levels allow anti-FVIII antibody-secreting plasma cells to survive and produce inhibitors in congenital hemophilia A patients with inhibitors. Despite such research, the typical presentation of BAFF levels in patients with AHA remains to be elucidated. Our preliminary measurement of BAFF in two patients with AHA revealed an elevated level of BAFF in one patient but a normal level in the other, suggesting that BAFF might be involved in the pathogenesis of AHA in at least some AHA patients. Although further study is warranted before its application in the clinical setting, the targeting of BAFF as a therapeutic strategy appears promising in the treatment of a subset of AHA patients, as well as of hemophilia A patients with refractory inhibitors presenting with elevated BAFF levels.

6. Diagnosis

6.1. Cross-Mixing Testing. The first step in diagnosis of AHA is tracking signs of bleeding tendency, particularly in the elderly, in the clinical setting and testing for prolongation of activated partial thromboplastin time (APTT) in the laboratory. The next step is review of patient medical history by consideration of the impact of any underlying conditions associated with AHA. APTT prolongation reflects decreased levels of coagulation intrinsic factors VIII and IX, as well as decreased levels of factors XI and XII, prekallikrein, and high molecular weight kininogen, which are involved in the contact system of coagulation. However, since reduction of proteins involved in the contact system is not associated with bleeding tendencies [44, 45], these conditions are ruled out in the differential diagnosis.

To diagnose AHA, measurement of FVIII:C is essential, and consecutive determination of inhibitor titer is a requisite in cases of decreased level of FVIII:C. While APTT is prolonged in patients with low levels of FVIII:C by anti-FVIII neutralizing autoantibodies, PT, fibrinogen and VWF levels, and platelet count are within normal limits and platelet function is normal. Since thrombocytopenia, PT and APTT prolongation, and decreased levels of fibrinogen are often observed in DIC patients who are erroneously diagnosed with AHA, consideration of these findings is helpful in differentiation of DIC from AHA.

Several cross-mixing studies have been performed to examine whether APTT prolongation results from a deficiency of intrinsic factor(s) or inhibitor. In one such study, addition of an equal volume of normal control plasma to patient's plasma was found to correct the APTT value to the normal range in coagulation-factor-deficient patients but not AHA patients [46]. As FVIII inhibition by autoantibodies is time- and temperature-dependent, the mixture in all such studies should be incubated at 37°C for 1 to 2 hours and, if correction of APTT value is unsuccessful, the presence of an inhibitor should be suspected. Recently, a cross-mixing test originally developed to differentiate lupus-anticoagulant presence from coagulation-factor deficiency has been established as a more useful laboratory test to determine the cause of APTT prolongation and thus useful in AHA diagnosis [47]. The plotting of the results of a cross-mixing test with altering the proportion of normal control plasma to the patient's plasma yields a convex APTT value curve that faces upward in the presence of inhibitors (including coagulation factor-neutralizing antibodies and lupus anticoagulants) and downward in the presence of a factor deficiency (Figure 3).

6.2. Lupus Anticoagulant. APTT is prolonged in the presence of lupus anticoagulants that interfere with the assembly and activity of the FXa-FVa-Ca^{2+} phospholipid complex. Lupus anticoagulants are polyclonal immunoglobulins that bind to phospholipids and proteins associated with the cell membrane and show nonspecific inhibitory effects that result in prolongation of both APTT and PT. From the perspective of laboratory testing, since intrinsic coagulation factor activity, including that of FVIII, appears to decrease in the presence of lupus anticoagulants, it is often difficult to distinguish AHA from lupus anticoagulants even by a mixing test. If the results of a mixing test indicate the presence of an inhibitor, the lupus anticoagulant is therefore evaluated by phospholipid-sensitive functional-coagulation assay, such

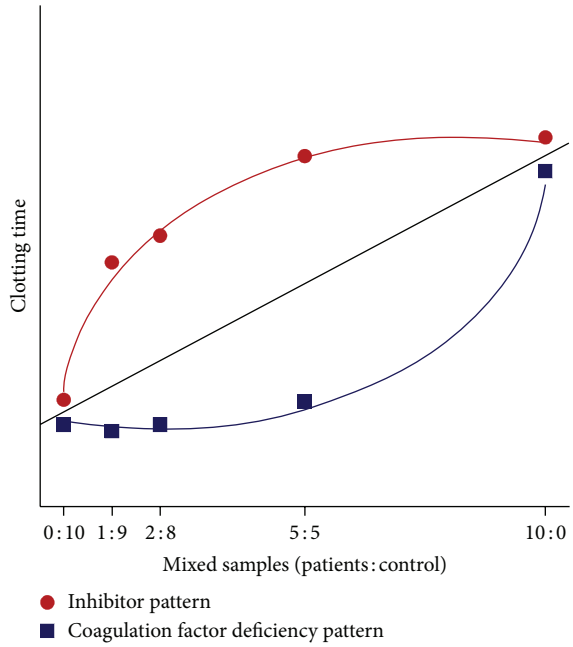

Figure 3: Cross-mixing test for detection of lupus anticoagulants or inhibitor of coagulation factor. A convex upward curve indicates the presence of inhibitors, including lupus anticoagulants and coagulation factor-neutralizing antibodies, while a convex downward curve indicates the presence of a factor deficiency.

as the dilute Russell's viper-venom time assay [48]. The coagulant in the venom directly activates FX, indicating that the dilute Russell's viper-venom time assay is dependent on a common pathway, including a pathway with phospholipids, and not influenced by deficiency or inhibition of intrinsic factors. Thus, the addition of exogenous phospholipids will correct the prolongation value as measured by clotting assay, confirming the presence of a lupus anticoagulant. One type of clotting test, the platelet neutralization procedure (PNP), takes advantage of the fact that lupus anticoagulants are absorbed onto the phospholipids on the surface of platelets while FVIII inhibitors are not absorbed [49]. The addition of washed platelets to the patient's plasma with lupus anticoagulant will thus decrease APTT prolongation.

6.3. Inhibitor Measurement. When the presence of an inhibitor is suspected, the targeted factor should be identified and the extent of inhibitory activity quantified. For the quantification of FVIII inhibitors, the Bethesda assay is the most commonly used laboratory test worldwide [50]. The classic Bethesda method measures the quantity of residual FVIII:C of a mixture containing equal amounts of normal control plasma and serially diluted patient plasma after incubation at 37°C for 2 h. The level of residual FVIII:C in the patient's plasma with inhibitors increases in tandem with the increasing dilution rate. The inhibitor titer value (1.0 BU/mL) used in the Bethesda assay is the reciprocal of the value of the dilution of the patient's plasma that leads to 50% inhibition. In the Nijmegen modification, which permits more accurate measurement of low titers of FVIII inhibitor, buffer is added to the Bethesda assay to maintain the sample plasma pH within the physiological range for the 2-hour incubation period and thereby stabilize FVIII in normal control plasma [51].

Although these assays are useful for determination of titers of alloantibodies against FVIII in congenital hemophilia A patients with type I kinetics, exact determination of autoantibody titer in AHA is difficult in patients with type II kinetics, in whom the acquired inhibitor-FVIII complex may show some residual FVIII:C, even in the presence of high concentrations of inhibitors. Therefore, measurement of levels of FVIII-binding antibodies is necessary for performing meaningful clinical assessment of the inhibitors present in AHA [13]. Prior to development of enzyme-linked immunosorbent assay (ELISA), measurement of FVIII-binding antibodies was traditionally performed using the agarose gel method [52, 53]. Previous studies have demonstrated that noninhibitory antibodies can be detected by ELISA in hemophilia patients in whom no inhibitors were detected using the Bethesda method [54–57]. In accordance with previous research into the use of the immunoprecipitation method for measurement of noninhibitory antibodies [58, 59], our investigation of the efficacy of immune-tolerance induction therapy in hemophilia A with refractory inhibitors using the immunoprecipitation method revealed that the method yields results of sufficient sensitivity [60]. However, use of all of these methods has certain drawbacks, such as the need to use radioactive materials and perform complex, time-consuming procedures. Fortunately, the ability of fluorescent microbeads method to overcome these drawbacks has been demonstrated in several studies [61], including one study in which we demonstrated its usefulness for assessing AHA patients as well as hemophilia A patients with inhibitors (Figure 4) [62]. As measurement using the fluorescent microbeads method is almost completely unaffected by the presence or absence of residual FVIII:C, use of the method allows for detection of antibodies without the undue influence of the presence of lupus anticoagulants or heparin.

7. Clinical Management

Favorable outcome in AHA depends on selection of an appropriate therapeutic approach based on early, correct diagnosis. The therapeutic strategy should aim for the achievement of 2 targets: control of bleeding and eradication of inhibitors.

7.1. Treatment of Acute Bleeding. Bleeding episodes in AHA are often severe and life threatening and presents with severe anemia. As massive subcutaneous or intramuscular hemorrhage may continuously worsen if left untreated, provision of immediate hemostatic therapy and monitoring of its efficacy by observation of improvement in anemia and clinical manifestations is required. The first-line treatment for severe bleeding episodes, especially in patients with high titers of inhibitors, is administration of bypassing agents [63, 64]. Activated prothrombin complex concentrates (APCC) containing factors II (prothrombin), VII, IX, and X or recombinant activated factor VII are commonly administered and have shown to be beneficial in treating patients with AHA as

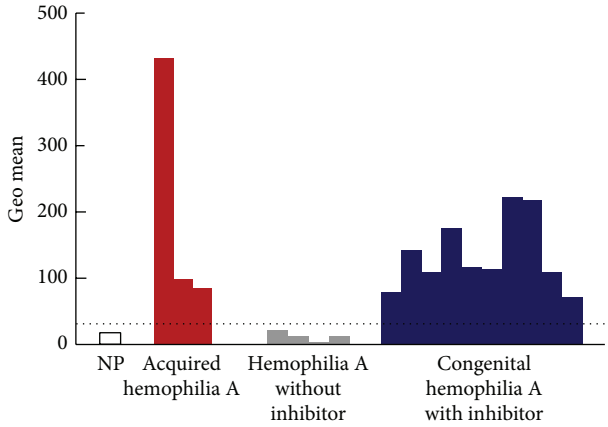

Figure 4: Flow cytometric analysis of factor VIII- (FVIII-) binding antibodies. Plasma samples from 20 normal healthy volunteers (normal pooled plasma), 3 acquired hemophilia A patients, 4 congenital hemophilia A patients without inhibitors, and 10 congenital hemophilia A patients with inhibitors were assessed using the following procedure. Human recombinant FVIII (rFVIII) was bound to red fluorescent carboxylated polystyrene microbeads (Cyto-Plex polystyrene microbeads) and a certain number of human rFVIII-bound microbeads were added to serially diluted suspected plasma. After incubation and washing, PE-labeled anti-human IgG antibody was added to the microbeads. After additional incubation and washing, fluorescent intensity was measured using a FACScan flow cytometer. The fluorescence intensity of the anti-human IgG antibody bound to human rFVIII on the microbead surface was expressed as the geometric mean (shown in arbitrary units). The dotted line shows a tentative cutoff value for the inhibitor with the highest geometric mean value in plasma without inhibitor. NP: normal plasma.

well as congenital hemophilia A patients with inhibitors [63–66]. Use of the immunoadsorption technique for removal of high-titer inhibitors has also proven beneficial in AHA patients with acute, life-threatening bleeding [67].

Another hemostatic treatment, the provision of inhibitor-neutralizing therapy with administration of FVIII concentrates at a level sufficient for neutralizing inhibitors, may also be beneficial for these patients. However, it is difficult to determine the quantity of FVIII required and calculate the half-life of the infused FVIII owing to the presence of type II inhibitors in AHA. In contrast, the requisite quantity of FVIII for neutralizing type I inhibitor in congenital hemophilia A can be determined theoretically. Therefore, frequent monitoring of hemostatic functioning accompanied by measurement of FVIII:C and/or APTT should be performed while providing neutralizing therapy to AHA patients. Administration of desmopressin, which stimulates the release of FVIII and VWF from endothelial cells and can provide a transient rise in FVIII:C levels to therapeutic levels [68], may also be effective in AHA patients with low titers of inhibitors or an FVIII:C level >5% [64]. While desmopressin has the advantages of being of low cost and safety, it does not entirely increase FVIII:C level to a therapeutic level and becomes less efficacious with repetitive administration. As neither therapy is adequate for AHA patients with high titers of inhibitors or severe bleeding symptoms, a bypassing strategy should be used with these patients.

7.2. Suppression of Inhibitor Formation. As with congenital hemophilia A with inhibitors, suppression and eradication of inhibitors are essential for normalization of hemostatic function and elimination of the risk of hemorrhage in AHA. For this, provision of immunosuppressive therapy is critical. In some cases of postpartum and drug-induced acquired hemophilia that resolves spontaneously, immunosuppressive therapy may be unnecessary [69]. However, even if bleeding symptoms are mild, the risk of severe and fatal hemorrhage persists unless inhibitors are eradicated. Therefore, immediate initiation of immunosuppressive therapy after confirmation of AHA diagnosis is recommended [70–73]. Several studies have established the effectiveness of immune-tolerance-induction therapy based on repetitive high-dose FVIII infusion for the eradication of inhibitors developed in congenital hemophilia A [74]. Immune-tolerance-induction methods that have been reported to be effective for treating AHA include not only administration of high-dose FVIII but also immunoadsorption and immune suppression therapy [75], the latter of which is likely essential for therapeutic success.

Agents used in immunosuppressive therapy for suppression of inhibitors include immunosuppressive agents such as prednisone, azathioprine, and cyclosporine and antineoplastic agents such as cyclophosphamide (CPA), mercaptopurine, and vincristine. Among these, administration of prednisone alone or in combination with CPA has been a common strategy. Combined prednisone-CPA administration has been reported to yield favorable outcomes [29, 64, 76], indicating that combined use of immunosuppressive or antineoplastic agents and prednisolone may yield beneficial effects. High-dose intravenous immunoglobulin therapy can be provided as an adjunctive therapy but should not be used as an initial treatment [12]. Physical removal of inhibitors by plasma exchange therapy or protein A adsorption column is effective for transient removal of inhibitors in patients with acute, severe bleeding [69]. Recently, several case studies of successful treatment with chimeric monoclonal antibodies targeted against the pan-B-cell marker CD20 (rituximab) in patients refractory to initial immunosuppressive therapy have been reported [77]. In cases where increased BAFF levels activate B-cells, use of a strategy to suppress B-cell activation appears rational.

There is no evidence that one immunosuppressive therapy is clinically superior to all others in treating AHA or that a certain therapy should be chosen depending on inhibitor titer or the hemorrhagic status. Therefore, first-line treatment is determined by evaluation of disease condition and consideration of possible adverse effects [64]. Although acute hemorrhage in AHA is potentially lethal, infectious diseases, such as pneumonia and sepsis, are responsible for approximately 50% of mortality associated with AHA [78]. Therefore, sufficient attention to prevention and early detection of infectious disease is warranted when aggressive and prolonged immune suppression therapy is provided.

8. Conclusions

AHA is characterized by the presence of an autoimmune mechanism that alone or accompanied by autoimmune disease, aging, pregnancy, or drug exposure causes breakdown of immune tolerance to FVIII associated with CD4 T-cells and results in development of autoantibodies against FVIII. In addition to treatment for acute bleeding, which is often required for AHA patients, immune suppression is essential for eradication of the inhibitors that play a central role in AHA pathogenesis. While provision of immunosuppression therapies, such as combined prednisone-CPA therapy, is currently the first-line treatment, administration of anti-CD20 monoclonal antibody (rituximab) appears to be a promising alternative treatment for AHA. Consideration of the findings regarding the association between the autoimmune mechanism responsible for AHA development and the innate immune system presented here and further elucidation of this association in future research will provide for better understanding of AHA pathophysiology and the development of novel therapies for eradication of inhibitors.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Isolated IgA Anti-β2 Glycoprotein I Antibodies in Patients with Clinical Criteria for Antiphospholipid Syndrome

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Seronegative antiphospholipid syndrome (SNAPS) is an autoimmune disease present in patients with clinical manifestations highly suggestive of Antiphospholipid Syndrome (APS) but with persistently negative consensus antiphospholipid antibodies (a-PL). IgA anti-β2 Glycoprotein I (aB2-GPI) antibodies are associated with APS. However, they are not currently considered to be laboratory criteria due to the heterogeneity of published works and the use of poor standardized diagnostic systems. We have aimed to assess aPL antibodies in a group of patients with clinical manifestations of APS (C-APS) to evaluate the importance of the presence of IgA aB2GPI antibodies in APS and its relation with other aPL antibodies. Only 14% of patients with C-APS were positive for any consensus antibody, whereas the presence of isolated IgA aB2GPI antibodies was found in 22% of C-APS patients. In patients with arterial thrombosis IgA aB2GPI, antibodies were the only aPL antibodies present. Serologic profile in primary APS (PAPS) is different from systemic autoimmune disorders associated APS (SAD-APS). IgA aB2GPI antibodies are more prevalent in PAPS and IgG aB2GPI antibodies are predominant in SAD-APS. The analysis of IgA aB2GPI antibodies in patients with clinical manifestations of PAPS might avoid underdiagnosed patients and provide a better diagnosis in patients with SAD-APS. Laboratory consensus criteria might consider including analysis of IgA aB2GPI for APS diagnosis.

1. Introduction

Antiphospholipid antibodies (aPL) are a heterogeneous group of autoantibodies directed against phospholipids, phospholipids complexed with proteins, or phospholipids binding proteins, localized on the membranes of endothelial cells, platelets, and other cells involved in the coagulation cascade [1, 2]. Antiphospholipid syndrome (APS) is an autoimmune multisystemic disorder characterized by recurrent thrombosis and pregnancy morbidity in patients with aPL antibodies [3]. APS was defined in the context of systemic autoimmune diseases as Systemic Lupus Erythematosus (SLE). However, shortly after, several authors suggested a separate category to group patients with APS clinical criteria and without systemic autoimmune disorders: the primary antiphospholipid syndrome (PAPS) [4, 5], currently the most common form of disease [6]. Patients with APS associated with systemic autoimmune disorders (also known as secondary antiphospholipid syndrome) were classified as SAD-APS [7]. Clinical criteria to diagnose APS include one or more

episodes of arterial, venous, or small vessel thrombosis in any tissue or organ that must be confirmed by objective validated criteria as imaging studies or histopathology. Consensus APS pregnancy morbidity can be (1) unexplained death of a morphologically normal fetus at or beyond the 10th week of gestation, (2) premature births of a morphologically normal neonate before the 34th week of gestation because of eclampsia or severe preeclampsia or placental insufficiency, and (3) three or more unexplained consecutive spontaneous abortions before the 10th week of gestation. Laboratory criteria are (1) presence of Lupus anticoagulant (LA) in serum or plasma, (2) presence of anticardiolipin (aCL) antibodies IgG and/or IgM isotype in serum or plasma, and (3) presence of anti-β_2 glycoprotein-I (aB2GPI) antibodies IgG and/or IgM isotype in serum or plasma. Antibodies should be present on two or more occasions at least 12 weeks apart. At least one clinical criterion and one laboratory criterion are needed for APS diagnosis [8]. Establishment of consensus criteria for APS allowed clinicians to standardize patient groups but also generated controversy. Several manifestations associated with antibodies aPL as heart valve disease, livedo reticularis (LR), aPL nephropathy, neurological manifestations, stroke, myocardial infarction, and thrombocytopenia were not included in the updated criteria [9, 10]. In addition, there are patients with clinical manifestations highly suggestive of APS but persistently negative for consensus aPL antibodies. These patients are classified as seronegative APS (snAPS) [11] and show similar clinical profile as seropositive patients [12].

In snAPS patients, recent works have revealed presence of aPL antibodies not included in APS criteria which might be relevant for the diagnosis of APS [13]. On the other hand, published aPL prevalence in the general population is highly heterogeneous, ranking between 1% and 5.6% in healthy subjects. Given these considerations, some authors have claimed that the current diagnostic criteria are too restrictive and of limited use for clinical purposes [14] and have suggested redefining APS [15].

Over the past few years much attention has been focused on the diagnostic value of IgA isotype aPL antibodies. Isolated IgA aB2GPI antibodies have been associated with APS on SLE patients [16] and with nonconsensus APS vascular pathology [17–19].

Although the majority of the published works have highlighted the value of IgA aB2GPI antibodies in APS diagnosis, there is controversy in the literature about the meaning of the presence of aB2GPI IgA antibodies. Insufficient standardization might be one of the causes and diagnostic tools are not sufficiently standardized. In addition, some works have been done with diagnostic assays that have not been optimized [20] that claim that IgA aB2GPI antibodies lack specificity in APS diagnosis and that do not provide useful clinical information. However, IgA aB2GPI antibodies have gained clinical relevance and were recently included as a clinic classification criterion for systemic lupus erythematosus [17]. Likewise, determination of IgA aB2GPI antibodies is recommended in patients with snAPS [21], LES, and in ethnics groups with a high prevalence of IgA isotype antibodies such as African Americans and long lasting SLE patients [18, 22].

Table 1: Clinical criteria of inclusion in APS patients group and prevalence.

Clinical criteria	Patients
Venous thrombosis	117 (75.0%)
Arterial thrombosis	11 (7.0%)
Venous and arterial thrombosis	2 (1.3%)
Pregnancy morbidity	25 (16.0%)
Venous thrombosis and pregnancy morbidity	1 (0.6%)

In this work, we have studied a group of patients with clinical manifestations of APS (C-APS) to determine the presence of aPL consensus isotypes (IgG and IgM) and also the IgA isotype in order to evaluate diagnostic utility of IgA isotype antibodies detection.

2. Patients and Methods

2.1. Study Design. This work is a cross-sectional study carried out to assess the prevalence of APS and snAPS conditions and their association with aPL antibodies of IgG, IgM, and IgA isotypes.

The study complies with Spanish legislation and European Community directives for cross-sectional studies.

2.2. Patients. A total of 156 patients fulfilling clinical criteria for APS (independently of serological markers) were recruited out of the 902 patients referred by their physicians to the Immunology Department in the 12 de Octubre Hospital during a 5-month period (ending on November 4, 2013). Presence of serum aPL antibodies was analyzed. Clinical criteria for patient inclusion (Table 1) were venous thrombosis (VT), arterial thrombosis (AT), and pregnancy morbidity (PM). Pulmonary thromboembolisms were classified as VT. Patients with incomplete symptoms of APS (livedo reticularis, thrombocytopenia, abortions outside deadlines, etc.) or prothrombotic conditions secondary to other factors such as sepsis, homocystinemia, and genetic defects of coagulation factors (thrombin mutations, factor V Leiden, antithrombin deficiency, etc.) were ruled out. Women with gestational morbidity were studied to evaluate the cause of this problem. Chromosomal, anatomical, endocrine, infectious, immune, and thrombophilic factors were studied. All those women who presented any of the above factors with the exception of aPL were excluded from the study.

Mean age of the patients was 52.3 ± 1.8 years. The proportion of women was approximately 2 : 1 (65.4%: 102 women, 54 men). Fifteen patients (9.4%) had associated systemic autoimmune diseases: SLE: 13 (8.3%), systemic sclerosis (SS): 1, and rheumatoid arthritis + SLE: 1. These 15 patients were considered as SAD-APS. The remaining 141 had no association with any systemic autoimmune disease and were considered PAPS. Additional risk factors found in the patients were diabetes mellitus 16 patients (10.3%), hyperlipidemia 24 patients (15.4%), hypertension 48 patients (30.8%), Chronic kidney disease 3 patients (1.9%), surgery within the previous

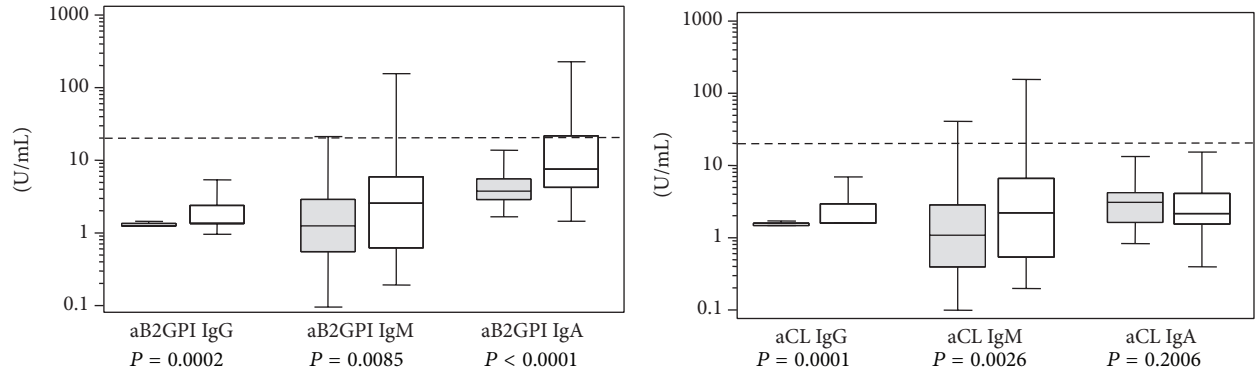

Figure 1: (a) Levels of anti-Beta 2 Glycoprotein I antibodies (aB2PGI) in controls (gray) and C-APS patients (white). (b) Levels of anticardiolipin antibodies (aCL) in controls (gray) and C-APS patients (white). Cutoff is indicated by a dotted line.

two months 3 patients (1.9%), and prolonged immobility 1 patient (0.6%).

Ethnicity of patients and controls was Mediterranean Caucasian in more than 97%.

2.3. Controls. The control group was randomly selected to represent the general population in our area. We included 306 sera from healthy blood donors, only considering one sample per patient because in our experience, a single determination of IgA aB2GPI antibodies has high diagnostic value [19]. In our laboratory, 3452 patients with at least 2 IgA aB2GPI determinations in the last 5 years were evaluated, 95.9% of whom had reproducible results.

Data of the patients and control were collected in an anonymized database. Sera samples were destroyed once the analysis was performed.

2.4. Laboratory Determinations. IgG/IgM aCL and aBGPI antibodies were measured using the BioPLex 2200 multiplex immunoassay system (Biorad, Hercules CA, USA). Antibody levels higher than 18 U/mL were considered positive following the manufacturer's guidelines.

IgA aCL and aBGPI antibodies were quantified by enzyme-linked immunosorbent assays (ELISA) using IgA-aCL and IgA-aB2GPI QUANTA Lite (INOVA Diagnostics Inc., San Diego, CA, USA). Antibody levels higher than 20 U/mL were considered positive following the manufacturer's guidelines.

Lupus anticoagulant (LA) activity was detected by coagulation assays, following the guidelines of the International Society on Thrombosis and Hemostasis (ISTH) [23]. We used the HemosIL dRVVT Screen, HemosIL dRVVT Confirm and HemosIL Silica Clotting Time assays (Instrumentation Laboratory SpA, Milano, Italy).

All serum samples were tested for IgG, IgM and IgA aCL and aB2GPI antibodies.

LA was determined in the 82 patients, which had been requested by their physicians (independently of APTT results). In addition, it was also determined in 8 additional patients who had APTT prolongation, although their physician had not requested it.

Table 2: Quantitative values (U/mL) of autoantibodies aPL in patients with symptoms of APS versus control population.

Antibody	Control		APS		
	Mean	S.E.	Mean	S.E.	*P* value
aB2GPI IgG	1.6	0.07	14.6	3.35	0.0002
aB2GPI IgM	2.6	1.87	7.6	0.30	0.0085
aB2GPI IgA	5.5	0.52	26.9	3.94	<0.0001
aCL IgG	2.0	3.28	14.8	0.10	0.0001
aCL IgM	2.2	0.20	6.4	1.34	0.0026
aCL IgA	3.5	0.95	8.2	3.20	0.2006

2.5. Statistical Methods. Results were expressed as mean ± standard error or absolute frequency and percentage. In scaled variables with two categories, comparisons were performed using the Student's *t*-test. Association between qualitative variables was determined with Pearson's Chi-square test or Fisher's exact test when appropriate. *P* values less than 0.05 were considered significant.

Data were processed and analyzed using the statistical program STATA 11 (StataCorp LP, College Station, TX, USA).

3. Results

3.1. aPL Antibody Levels and Their Relationship with C-APS Patients. Mean levels of IgG, IgM, and IgA, both aCL and aB2GPI, antibodies were significantly higher in patients with C-APS than in controls (Table 2, Figures 1(a) and 1(b)).

Proportion of patients with aCL or aB2GPI antibodies of any isotype was significantly higher in C-APS patients than in controls (Table 3). IgA aB2GPI was the most prevalent antibody in C-APS patients (28.8%, Table 3). The main difference between C-APS patients and controls was found in IgA aB2GPI antibodies positivity, combined with any other aPL (odds ratio 24.4 $P < 0.0001$) or isolated (odds ratio 17.4 $P < 0.0001$, Table 3). On the C-APS group, only 22 patients (14.1%) were positive for any consensus aPL (IgG/IgM aCL or aB2GPI antibodies). Thirty-five patients (22.4%) were positive for isolated IgA aB2GPI antibodies and 45 patients

Table 3: Positive aPL antibodies in C-APS patients versus controls.

	Controls (n = 306)	APS (n = 156)	OR	P
aB2GPI IgG	2 (0.6%)	14 (8.9%)	14.9	<0.0001
aB2GPI IgM	3 (0.9%)	8 (5.1%)	5.4	0.0146
aB2GPI IgA	5 (1.6%)	45 (28.8%)	24.4	<0.0001
aCL IgG	3 (0.9%)	17 (10.8%)	12.3	<0.0001
aCL IgM	2 (0.6%)	8 (5.1%)	8.2	0.0053
aCL IgA	3 (0.9%)	8 (5.1%)	5.5	0.0148
aB2GP1 IgA (isolated)	5 (1.6%)	35 (22.4%)	17.4	<0.0001
aCL or aB2GPI (IgG or IgM)	6 (2.0%)	22 (14.1%)	8.2	<0.0001
aCL or aB2GPI any isotype	13 (4.2%)	61 (39.1%)	14.5	<0.0001

Table 4: Positive aPL antibodies in PAPS versus SAD-APS patients.

Antibody	PAPS (n = 141)	SAD-APS (n = 15)	OR SAD-APS	P
aB2GPI IgG	3 (2.1%)	11 (73.3%)	126.5	<0.0001
aB2GPI IgM	5 (3.5%)	3 (20%)	6.8	0.0331
aB2GPI IgA	36 (21.9%)	9 (60%)	4.4	0.0124
aCL IgG	6 (4.2%)	11 (73.3%)	61.9	<0.0001
aCL IgM	5 (3.5%)	3 (20%)	6.8	0.0331
aCL IgA	4 (2.8%)	4 (26.7%)	12.4	0.0008
aB2GP1 IgA (isolated)	33 (23.4%)	2 (13.3%)	0.5	0.5732
aCL or aB2GPI (IgG or IgM)	11 (7.8%)	11 (73.3%)	32.5	<0.0001
aCL or aB2GPI any isotype	47 (33.3%)	14 (93.3%)	28.0	<0.0001

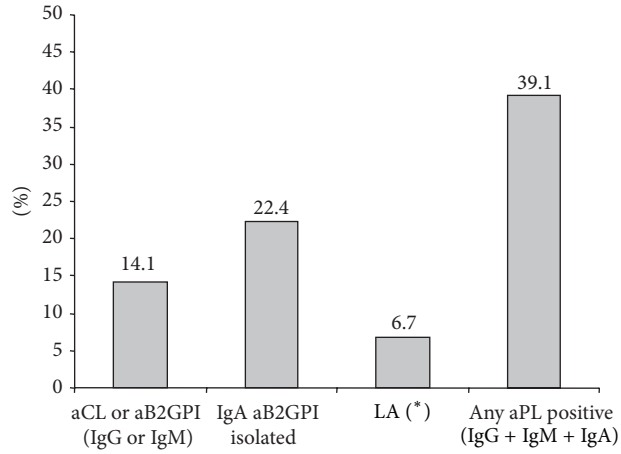

Figure 2: Percentage of C-APS patients positive for aPL antibodies. (*) LA detection was performed on 90 patients.

(28.8%) were positive for IgA B2GPI antibodies combined with other isotypes (Table 3).

If we consider those with positivity of any aPL isotype antibodies (including IgA) as aPL positive patients, 61 patients would be positive (39.1%) due to the inclusion of 39 new patients who were positive for IgA isotype and negative for IgG and IgM (Figure 2).

Lupus anticoagulant was only positive for 6 of the 90 patients tested (6.7%, Figure 2). No significant associations with previously described risk factors were observed (not shown).

3.2. Prevalence of aPL Antibodies in PAPS and SAD-APS. SAD-APS patients were younger than PAPS ones (44.3 ± 3.0 versus 56.2 ± 1.7 years, P = 0.0021), with a greater percentage of women (93.3% versus 62.4%, P = 0.0200).

Positivity of consensus aPL antibodies in SAD-APS patients was significantly higher than in patients with PAPS (Table 4, Figures 3(a) and 3(b)), especially for IgG isotype antibodies with odds ratios higher than 60 (P < 0.0001, Table 4). Positivity of IgA aB2GPI antibodies combined with other consensus aPL antibodies was also higher in SAD-APS patients (P = 0.0124) but isolated positivity of IgA aB2GPI antibodies did not show significant differences with PAPS group (P = 0.5732, Table 4).

Eleven (7.8%) PAPS patients were positive for consensus aPL antibodies isotypes. When IgA isotype positivity was also considered, 33.3% of the patients were seropositive (Figure 3(a)).

The most prevalent antibodies on PAPS patients were IgA aB2PGI (Table 4). Isolated IgA aB2GPI were the only positive antibodies in 70% of these seropositive patients.

Eleven (73.3%) of SAD-APS patients were positive for consensus isotypes aPL antibodies. When IgA isotype were included, 93.3% of patients were identified as seropositive. This improves the diagnostic capacity of consensus aPL but more discretely than in PAPS patients (Figure 3(b)). The most prevalent antibodies in SAD-APS patients were IgG aB2PGI (Table 4). No significant differences were observed between PAPS and SAD-APS patients regarding the clinical classification inclusion criteria (not shown).

Table 5: APS morbidity and aPL autoantibodies.

Antibodies	Venous thrombosis			Arterial thrombosis			Pregnancy morbidity		
	N	OR	*P*	*N*	OR	*P*	*N*	OR	*P*
aB2GPI IgG	12 (10%)	16.9	<0.0001	0 (0%)	0	0.1332	2 (8%)	12.6	0.0262
aB2GPI IgM	7 (6%)	6.2	0.0087	0 (0%)	0	0.2677	1 (4%)	4	0.7265
aB2GPI IgA	36 (30%)	25.8	<0.0001	7 (54%)	70.2	<0.0001	3 (12%)	7.8	0.0125
aCL IgG	15 (13%)	14.4	<0.0001	0 (0%)	0	0.2677	2 (4%)	8.4	0.0630
aCL IgM	7 (6%)	9.4	0.0029	0 (0%)	0	0.1332	1 (8%)	6	0.5671
aCL IgA	7 (6%)	6.2	0.0087	1 (0%)	0	0.2677	1 (4%)	4	0.7265
aB2GP1 IgA (isolated)	28 (23%)	18.3	<0.0001	6 (46%)	51.6	<0.0001	2 (8%)	5	0.1759
aCL or aB2GPI (IgG or IgM)	20 (17%)	10	<0.0001	0 (0%)	0	0.4994	2 (8%)	4.1	0.2445
aCL or aB2GPI any isotype	50 (42%)	16.1	<0.0001	7 (54%)	26.2	<0.0001	5 (19%)	5.3	0.0053

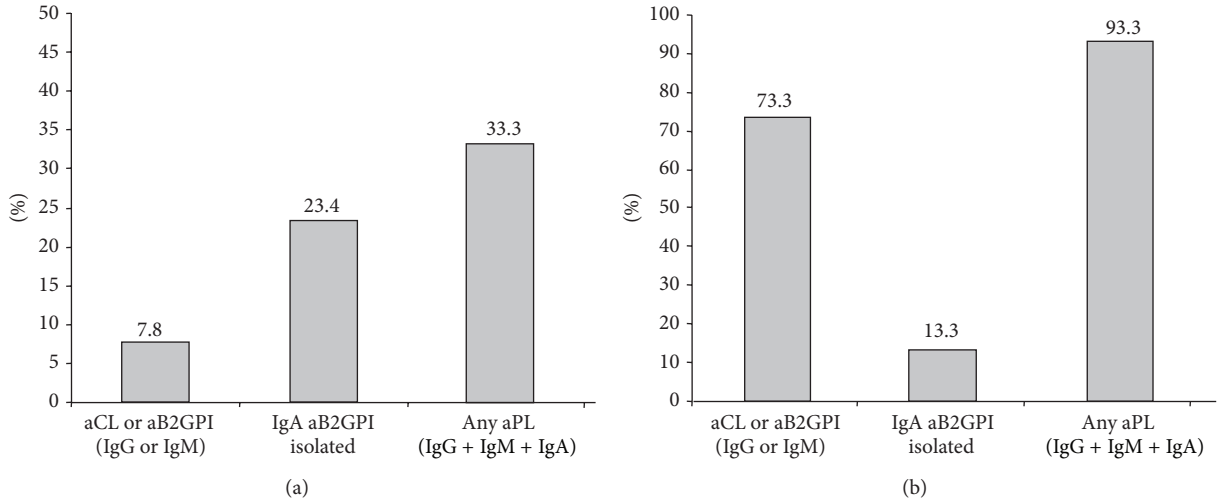

Figure 3: (a) Percentage of PAPS patients positive for aPL antibodies. (b) Percentage of SAD-APS patients positive for aPL antibodies.

3.3. Relationship between Clinical Manifestations of APS and aPL Antibodies. No differences between APS subgroups (VT, AT, and PM) were observed on aPL antibodies positivity (not shown). However, in patients with AT, IgA isotype antibodies were especially significant: 54% of the patients with AT were positive for IgA (odds ratio 70.2, $P < 0.0001$) and all patients with AT were negative for aPL antibodies of IgG and IgM isotypes (Table 5, Figure 4).

4. Discussion

Assessment of IgA isotype aPL antibodies, especially anti B2GPI, allowed clinicians to identify more patients with C-APS as seropositive [24], detecting up to nearly 40% of the cases while using Sapporo's consensus criteria of laboratory diagnosis only detected 14.1% of the cases.

The prevalence of aPL autoantibodies in the control group was similar to previously reported studies for IgG and IgM isotype [9, 25] and also for IgA isotype [26]. Most patients positive for IgA anti B2GPI antibodies were negative for IgA aCL. The proportion of IgA aB2GPI positive versus IgA aCL positive was also similar to that previously published [16].

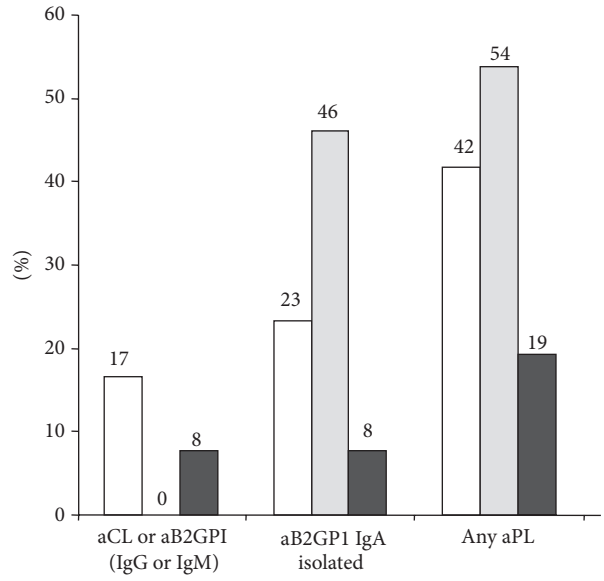

Figure 4: Percentage of APS patients positive for aPL antibodies. APS patients were classified as follows: venous thrombosis (white), arterial thrombosis (grey), and pregnancy morbidity (dark).

It stands out that only 9.4% of our patients with APS symptoms had SAD-APS when could be expected close to 50% according to the published data [6]. A possible explanation for this difference is because we were studying patients with C-APS and the published studies have only evaluated seropositive APS patients. If we limit our study only to the 22 patients positive for consensus aPL antibodies, patients with SAD-APS would be 50% (11), this being in accordance with the expected prevalence. This observation emphasizes that the laboratory criteria for APS were designed to achieve greater specificity in the SAD-APS, resulting in the disadvantage that cases of PAPS remain underdiagnosed [27].

The aPL antibodies profile differs for PAPS patients than for SAD-APS patients. Whereas in SAD-APS patients the most prevalent antibody is IgG isotype (aB2GPI and aCL), it is the IgA isotype in PAPS patients. Diagnostic utility of isolated IgA aB2GPI antibodies in patients with C-APS was previously reported in a small cohort of patients [22]; Our study has been carried out with a larger number of patients without any selection bias.

Incorporating the IgA isotype into the diagnostic guidelines could be especially useful in patients with PAPS. It would make it possible to identify up to 4 times more patients who are not considered as APS with the current diagnosis criteria. This change in criterion might be a diagnostic improvement, especially for patients with AT [28] who are negative for consensus aPL antibodies in our study.

If IgA isotype antibodies are taken into consideration as consensus aPL antibodies, about 27% more patients would be identified as SAD-APS. Although IgA isotype is less relevant in SAD-APS than PAPS, it has greater utility than that provided by the IgM isotype, as was observed previously [18].

The relevance of IgA aB2GPI antibodies in patients with SLE was recently accepted as an inclusion diagnostic criterion for SLE by the Systemic Lupus International Collaborating Clinics Classification Criteria for Systemic Lupus Erythematosus [17]. It is unknown how the immune response of IgA antibodies against B2GPI is generated. It has been hypothesized that anti-B2GPI response could occur by molecular mimicry between pathogens and B2GPI epitopes [3, 29] or within the context of an autoimmune syndrome induced by adjuvants (ASIA) [30]. If infection or antigen presentation takes place in the respiratory or digestive tracts, the mucosal immune system directs the response of the antibodies towards the production of the IgA isotype.

The mechanisms by which IgA aB2GPI antibodies can cause thrombosis remain unknown. Several pathogenic pathways have been suggested as hypercoagulable state secondary to activation of the complement system [31], inhibition of the fibrinolytic system [32], and cellular activation of platelets, monocytes, and endothelial cells (EC) [27, 33]. B2GPI is localized on the level of the cell surface of human EC associated with lipids or membrane proteins [34]. aB2GPI antibodies can activate EC [35], upregulate adhesion molecules, and induce cytokine production [36]. Cell activation by aPL antibodies appears to be a major pathogenic cause in the pathogenesis of APS [37]. As human IgA cannot fix the complement using the classical pathway [38], in the case of patients with isolated positivity of IgA aB2GPI this mechanism takes on special relevance.

If we consider the presence of IgA B2GPI antibodies as laboratory diagnostic criteria together with IgG and IgM antibodies, we can increase the number of APS patients diagnosed. However, 61% of C-APS patients could not be identified as seropositive.

In the near future, the determination of other less prevalent APL antibodies, as antiAnnexin V and antiphosphatidylserine/Prothrombin should be evaluated in order to identify more APS patients who are currently misdiagnosed.

Antibodies aB2GPI of IgA isotype are present in 1–3% of the healthy population. Even though these antibodies could be considered as an epiphenomenon, their importance has not been established yet. Prospective studies are needed to clarify their predictive value in vascular and thromboembolic events.

Most studies of aPL are made on series of patients with systemic autoimmune diseases (SAD-APS) and tend to extrapolate their conclusions to all patients with APS.

In this study we have focused on patients with clearly defined C-APS independently of the presence or not of underlying systemic autoimmune diseases. Therefore, we have been able to obtain a better idea of the importance of the C-APS in the clinical practice.

These results should be confirmed in multicenter studies that make it possible to manage larger groups of patients and would help to assess other APS-associated manifestations.

Our studies suggest that the serological profile of patients with PAPS (IgA is the most prevalent isotype) is different from the SAD-APS (IgG is the most prevalent isotype). The assessment of IgA aB2GPI antibodies in patients with suspected PAPS is important to identify, treat, and manage patients who, in accordance with the current criteria, are not diagnosed at this point in time of the disease. This is not as important as PAPS in the case of SAD-APS because patients are monitored regularly in the context of their underlying disease and any clinical event is quickly detected.

We agree with other authors that the classification criteria for APS should be revised to include IgA aB2GPI antibodies in patients with SLE [18] but perhaps it is even more important to include these criteria in PAPS.

Conflict of Interests

The authors declare they have no conflict of interests in this study.

Authors' Contribution

Raquel Ruiz-García and Manuel Serrano collaborated equally to this work.

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Research Article

The Mosaic of "Seronegative" Antiphospholipid Syndrome

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In the clinical practice it is possible to find patients with clinical signs suggestive of antiphospholipid syndrome (APS), who are persistently negative for the laboratory criteria of APS, that is, anti-cardiolipin antibodies (aCL), anti-β_2-GPI antibodies and lupus anticoagulant. Therefore, it was proposed for these cases the term of seronegative APS (SN-APS). In order to detect autoantibodies with different methodological approaches, sera from 24 patients with SN-APS were analysed for anti-phospholipid antibodies using TLC immunostaining, for anti-vimentin/cardiolipin antibodies by enzyme-linked immunosorbent assay (ELISA), and for anti-annexin V and anti-prothrombin antibodies by ELISA and dot blot. Control groups of our study were 25 patients with APS, 18 with systemic lupus erythematosus (SLE), and 32 healthy controls. Results revealed that 13/24 (54.2%) SN-APS sera were positive for aCL (9 of whom were also positive for lysobisphosphatidic acid) by TLC immunostaining, 11/24 (45.8%) for anti-vimentin/cardiolipin antibodies, 3/24 (12.5%) for anti-prothrombin antibodies, and 1/24 (4.2%) for anti-annexin V antibodies. These findings suggest that in sera from patients with SN-APS, antibodies may be detected using "new" antigenic targets (mainly vimentin/cardiolipin) or methodological approaches different from traditional techniques (mainly TLC immunostaining). Thus, SN-APS represents a mosaic, in which antibodies against different antigenic targets may be detected.

1. Introduction

Antiphospholipid antibody syndrome (APS) is characterized by arterial and/or venous thromboses, recurrent abortions or foetal loss, and circulating antiphospholipid antibodies (aPL) [1, 2]. According to the classification criteria, diagnosis of APS requires the combination of at least one clinical and one laboratory criterion [3, 4]. Anti-CL and anti-β_2-glycoprotein-I (aβ_2-GPI) antibodies, detected by enzyme linked immunosorbent assay (ELISA) and the lupus anticoagulant (LA) detected by clotting assays are the recommended tests for the detection of aPL [5, 6]. Nevertheless, in daily clinical practice it is possible to find patients with clinical signs suggestive of APS who are persistently negative for the routinely used aCL, aβ_2-GPI, and LA. Therefore, it was proposed for these cases the term of "seronegative APS" (SN-APS) [2, 7]. Three possible explanations for the existence of such "seronegative" cases have been proposed: either the diagnosis is wrong, or that previously positive aPL tests have become negative, or, as seems most likely, the current range of tests is inadequate [8].

Indeed, aPL represent a heterogeneous family of antibodies reacting with phospholipid-binding cofactor proteins, including not only β_2-GPI [9, 10] but also different anionic phospholipids, proteins, or phospholipid-protein complexes, such as prothrombin [11], protein S [12, 13], protein C [14], annexin V [15], annexin II [16], oxidized low-density lipoprotein, lysobisphosphatidic acid (LBPA), and sulfatides [17–19].

Recently, with a proteomic approach, we identified vimentin/cardiolipin as a "new" target of the APS, also detectable in SN-APS patients [20]. In addition, we demonstrated the possibility of detecting aPL in SN-APS patients by immunostaining on thin-layer chromatography (TLC) plates [21, 22]. This latter nonquantitative technique identifies the

reactivity of serum aPL with phospholipid molecules but with a different antigenic exposure as compared to ELISA method.

The aim of this study was to identify the best screening combination of "new" antigenic targets or methodological approaches to detect aPL in SN-APS patients.

2. Materials and Methods

2.1. Patients. The study included 24 consecutive patients, attending the Lupus clinic, Rheumatology Unit of the Sapienza University of Rome, presenting clinical features consistent with a diagnosis of APS but tested persistently negative (at least 2 times 12 weeks apart) for conventional aCL, aβ_2-GPI, and LA tests. Clinical manifestations included venous and/or arterial thrombosis and pregnancy morbidity as stated in the classification criteria for definite APS [3, 4]. Sera were collected at several times and stored at −20°C until use. Moreover, all patients showed normal screening for other causes of thrombophilia such as antithrombin, protein C and protein S deficiency, hyperhomocysteinemia, factor V, and prothrombin mutations.

Moreover, in this study, 43 consecutive out-patients, attending the Rheumatology Division of Sapienza University of Rome, were also included. Twenty-five had APS, diagnosed according to the Sapporo criteria [4]; they included both primary APS ($N = 9$) and APS associated with SLE ($N = 16$); 18 had SLE fulfilling the ACR revised criteria for the classification of SLE [23]. Finally 32 healthy subjects (normal blood donors) matched for age and sex were studied as controls.

This study was approved by the local ethic committees and participants gave written informed consent.

2.2. ELISA for aPL and Antiphospholipid-Binding Proteins. aCL and aβ_2-GPI ELISA kits were obtained from INOVA Diagnostics Inc. (San Diego, CA, USA). ELISA was performed according to the manufacturer's instructions. Antiannexin V and antiprothrombin were performed as previously described [11, 15] and confirmed by dot blot. A positive control and several normal human sera were run in the same assay to confirm the specificity of the results.

2.3. LA Test. LA was studied in two coagulation systems, a dilute sensitized activated partial thromboplastin time (aPTT) and a dilute Russell's viper venom time (dRVVT), followed by confirm test, using reagents and instrumentation by Hemoliance Instrumentation Laboratory, Lexington, MA, USA.

2.4. Detection of aPL by TLC Immunostaining. Immunostaining on TLC was performed as previously described, with slight modification [21, 22, 24, 25]. CL (Sigma Chemical Co., St Louis, MO, USA) and LBPA (Avanti Polar Lipids, Alabaster, AL, USA), 2 μg, were run on aluminium-backed silica gel 60 (20 × 20) high performance thin layer chromatography (HPTLC) plates (Merck Co, Inc Darmstadt, Germany). Chromatography was performed in chloroform : methanol : CH_3COOH : water (100 : 75 : 7 : 4) (v : v : v : v). The dried chromatograms were soaked for 90 sec in a 0.5% (w : v) solution of poly(isobutyl methacrylate) beads (Polysciences, Inc. Eppelheim, Germany) dissolved in hexane. After air-drying, the chromatograms were incubated at room temperature for 1 h with 1% BSA in PBS to eliminate nonspecific binding. The blocking solution was removed and replaced by a washing buffer (phosphate-buffered saline, PBS). The chromatograms were then incubated for 1 h at room temperature with sera, diluted 1 : 100 in the blocking solution. Sera were removed and chromatograms were washed 3 times for 10 min with PBS. Bound antibodies were visualized with HRP-conjugated goat anti-human IgG (Sigma-Aldrich, St Louis, MO, USA), diluted 1 : 1000 in 1% bovine serum albumin (BSA) in PBS, and incubated at room temperature for 1 h and immunoreactivity was assessed by the chemiluminescence reaction using the ECL Western blotting system (Amersham Pharmacia Biotech, Buckinghamshire, UK).

2.5. Detection of Antivimentin/Cardiolipin by ELISA. Antivimentin/cardiolipin complex antibodies were detected by a slight modification of ELISA method previously reported [20]. Polystyrene plates (96-well) were coated and incubated overnight at 4°C with 100 μL/well of cardiolipin (50 μg/mL; Sigma-Aldrich) in methanol and then with 100 μL/well of human recombinant vimentin (5 μg/mL; R&D System, Minneapolis, MN, USA) in 0.05 M $NaHCO_3$ buffer, pH 9.5. Coated plates were incubated overnight at 4°C and then washed 3 times with phosphate-buffered saline containing 0.1% Tween 20 (PBS-T). Plates were blocked for 2 h at room temperature with 100 μL of 1% BSA in PBS. After washing 3 times with PBS-T, the wells were incubated for 1 h at room temperature, with 100 μL of patient sera, diluted 1 : 100 in the blocking buffer. Each serum was analyzed in triplicate. Goat polyclonal antivimentin (R&D Systems) was used as a positive control.

After 3 washes with PBS-T, the plates were incubated for 1 h at room temperature with horseradish peroxidase-conjugated antibodies; either goat anti-human IgG or rabbit anti-goat IgG (Sigma-Aldrich) was diluted in 1% BSA in PBS. The plates were washed 3 times with PBS-T; the bound peroxidase was then revealed with 100 μL of *O*-phenylenediamine dihydrochloride and color development was stopped with H_2SO_4 0.2 M for 5 min. Absorbance was measured at 492 nm in a microplate reader. Data were presented as the mean optical density (OD) corrected for background (wells without coated antigen). Thirty-two normal human sera were also tested, and a cut-off value was established at a mean of optical density (OD) ± 3 SD of normal human sera. Parallel experiments were performed in which all the procedures were identical without coated vimentin/cardiolipin complex. Virtually no reactivity was detected in all the samples (data not shown).

3. Results

3.1. Characteristics of Patients. All but one (a 39-year-old female with Asian ethnicity) SN-APS patients enrolled in this

Table 1: Clinical characteristics of patients studied.

Characteristics *n* (%)	SN-APS (*n* = 24)	APS (*n* = 25)
Other autoimmune diseases	17 (70.8)	16 (64)
SLE	9 (37.5)	16 (64)
Sjögren syndrome secondary to SLE	2 (8.3)	8 (32)
Primary Sjögren syndrome	1 (4.2)	0
Rhupus	1 (4.2)	0
Mixed connective tissue disease	1 (4.2)	0
Undifferentiated connective tissue	1 (4.2)	0
Disease	1 (4.2)	0
Urticarial vasculitis	1 (4.2)	0
LED	1 (4.2)	0
Myasthenia gravis	0	1 (4)
None	5 (20.8)	9 (36)
Vascular thrombosis	18 (75)	24 (96)
Venous thrombosis	10 (41.6)	17 (68)
Arterial thrombosis	12 (50)	10 (40)
Recurrent thrombosis	8 (33.3)	10 (40)
Pregnancy morbidity	8 (33.3)	9 (36)
Normal fetus deaths	7 (29.2)	2 (8)
Premature births	0	0
Spontaneous abortions	5 (20.8)	8 (32)
Vascular thrombosis and pregnancy morbidity	3 (12.5)	6 (24)
Noncriteria APS features		
Livedo reticularis	5 (20.8)	7 (28)
Thrombocytopenia	1 (4.2)	5 (20)
Cognitive dysfunctions	1 (4.2)	6 (24)
Migraine	5 (20.8)	8 (32)
Seizures	0	5 (20)
Brain MRI scan abnormalities	10 (41.6)	8 (32)
Thrombotic risk factors	15 (62.5)	14 (56)
Hypercholesterolemia	5 (20.8)	10 (40)
Smoking	12 (50)	5 (20)
Hypertension	6 (25)	7 (28)
OC/HRT	2 (8.3)	2 (8)
Diabetes mellitus	1 (4.2)	0

study were Caucasian, 18 females and 6 males with a mean age of 45.7 years (range 26–82), and a mean disease duration of 9.3 years (range 0.8–57). The clinical characteristics of SN-APS patients are reported in Table 1. APS patients (3 males and 22 females) showed a mean age of 43.9 years (range 27–71) and a mean disease duration of 9.6 years (range 0.1–34). SLE patients were all females with a mean age of 36.8 years (range 18–59) and a mean disease duration of 13.4 years (range 0.8–36). None of the healthy subjects experienced arterial or venous thrombosis nor recurrent fetal loss.

A statistically significant correlation was found between arterial and/or venous thrombosis and pregnancy morbidity in SN-APS ($P < 0.001$).

3.2. Detection of aPL by TLC Immunostaining. In SN-APS patients the results obtained by TLC immunostaining showed the presence of aCl in 13 out of 24 patients (54.2%); anti-LBPA antibodies in 9 out of 24 (37.5%). All sera positive for anti-LBPA were also positive for aCL.

In APS patients TLC immunostaining showed the presence of antibodies against CL in 17/25 (68%), against LBPA in 14/25 (56%). In SLE patients TLC immunostaining showed the presence of antibodies against CL in 11/18 (61.1%), against LBPA in 11/18 (61.1%). Finally, none of the healthy subjects showed aPL reactivity by TLC immunostaining (Table 2).

3.3. Detection of aPL and Antiphospholipid-Binding Proteins by ELISA. Eleven out of 24 SN-APS patients (45.8%) showed serum antibodies (IgG class) against vimentin/cardiolipin, 3 (12.5%) against prothrombin, and 1 (4.2%) against annexin V (Table 2). None resulted positive for antibodies against CL or β_2-GPI.

In APS patients anti-vimentin/CL antibodies were detected in 22/25 (88%), antiprothrombin in 9/25 (36%), and antiannexin V in 14/25 (56%). Anti-CL reactivity was observed in 25/25 (100%). Anti-β_2-GPI reactivity was observed in 18/25 (72%) APS.

In SLE patients anti-vimentin/CL antibodies were detected in 7/18 (38.8%), anti-prothrombin in 1/18 (5.5%), and antiannexin V in 4/18 (22.2%). Moreover, anti-CL reactivity was observed in 14/18 (77.7%) and anti-β_2-GPI reactivity was observed in 7/18 (38.8%) SLE patients.

Finally, none of the 32 healthy subjects displayed positivity for the autoantibodies under test.

3.4. Associations between Autoantibodies and Clinical Features. Taken together, our findings show that in 19 out of 24 SN-APS (79.2%) at least one aPL/cofactor antibody was detected using the assays under test (Table 3).

Table 4 shows the prevalence of the autoantibodies in SN-APS patients with different clinical manifestations. No significant association was found between the prevalence of the clinical features in SN-APS patients and specific autoantibodies. The combination of two of the tested methodological approaches, TLC immunostaining for aCL and ELISA for anti-vimentin/cardiolipin complex antibodies, was able to detect aPL/cofactors in about two-thirds of SN-APS patients with thrombosis or pregnancy morbidity, with a small additional gain when also performing ELISA for prothrombin and annexin V (Table 4).

4. Discussion

In the clinical practice it is possible to find patients with clinical signs suggestive of APS, who are persistently negative for the routinely used assays to detect aCL, aβ_2-GPI and LA. For these cases the term of SN-APS has been proposed [26–30]. Similarly to classical APS, SN-APS can have an accelerated progression, resulting in multiorgan failure, ending to catastrophic APS [31].

Thus, since clinical features of SN-APS appear to be similar to APS, the most convincing explanation for the

Table 2: Occurrence of autoantibodies in SN-APS and control sera.

Autoantibodies	SN-APS (24) *n* (%)	APS (25) *n* (%)	SLE (18) *n* (%)	Healthy donors (32) *n* (%)
Anticardiolipin by TLC-immunostaining	13 (54.2)	17 (68)	11/18 (61.1)	0 (0)
Antivimentin/Cardiolipin	11 (45.8)	22 (88)	7/18 (38.8)	0 (0)
Antiprothrombin	3 (12.5)	9 (36)	1/18 (5.5)	0 (0)
Antiannexin V	1 (4.2)	14 (56)	4/18 (22.2)	0 (0)

Table 3: Positivity of autoantibodies in the 24 SN-APS sera.

Patient *n*	aCL by TLC immunostaining	Antivimentin/CL	Antiprothrombin	Antiannexin V
1	**Pos**	**Pos**	Neg	Neg
2	**Pos***	**Pos**	Neg	Neg
3	**Pos***	**Pos**	Neg	Neg
4	**Pos***	Neg	Neg	Neg
5	**Pos**	**Pos**	Neg	Neg
6	**Pos***	Neg	Neg	Neg
7	**Pos***	Neg	Neg	Neg
8	**Pos**	**Pos**	Neg	Neg
9	**Pos***	**Pos**	Neg	Neg
10	**Pos**	Neg	Neg	Neg
11	**Pos***	**Pos**	Neg	Neg
12	**Pos***	Neg	Neg	Neg
13	**Pos***	Neg	Neg	Neg
14	Neg	Neg	Neg	Neg
15	Neg	Neg	**Pos**	**Pos**
16	Neg	Neg	**Pos**	Neg
17	Neg	**Pos**	**Pos**	Neg
18	Neg	Neg	Neg	Neg
19	Neg	**Pos**	Neg	Neg
20	Neg	**Pos**	Neg	Neg
21	Neg	**Pos**	Neg	Neg
22	Neg	Neg	Neg	Neg
23	Neg	Neg	Neg	Neg
24	Neg	Neg	Neg	Neg

*Also positive for anti-LBPA.

Table 4: Autoantibody prevalence in seronegative antiphospholipid syndrome (SN-APS) patients ($n = 24$) according to the clinical manifestations.

Autoantibodies (assay)	Total thrombosis ($n = 18$)	Arterial thrombosis ($n = 12$)	Venous thrombosis ($n = 10$)	Recurrent thrombosis ($n = 8$)	Pregnancy morbidity ($n = 8$)
aCL (TLC)	11 (61.1%)	7 (58.3%)	6 (60.0%)	5 (62.5%)	3 (37.5%)
Antivimentin/CL	7 (38.9%)	4 (33.3%)	5 (50.0%)	3 (37.5%)	5 (62.5%)
aCL (TLC) + antivimentin/CL	12 (66.7%)	8 (66.7%)	6 (60.0%)	5 (62.5%)	5 (62.5%)
Antiannexin V	0	0	0	0	1 (12.5%)
Antiprothrombin	1 (5.6%)	0	1 (10.0%)	1 (12.5%)	2 (25.0%)
aCL (TLC) + antivimentin/CL + anti-annexin V + antiprothrombin	13 (72.2%)	8 (66.7%)	7 (70.0%)	6 (75.0%)	7 (87.5%)
No autoantibodies	5 (27.8%)	4 (33.3%)	3 (30.0%)	2 (25.0%)	1 (12.5%)

existence of such "seronegative" patients may be that the current range of tests is inadequate. It may depend either on limits of the traditional technical approaches or on the existence of different antigenic targets.

Considering the first possibility, we employed a different methodological approach for detection of aPL, TLC immunostaining, which relies upon the different partition characteristics of phospholipids between the surface (stationary phase) and mobile solvent phase for different solvent polarities. TLC immunostaining is useful for detection of aPL in the presence of cofactor proteins, mainly β_2-GPI (provided with the medium). However, in this case, the binding of phospholipid to solid phase mainly involves both electrostatic and hydrophobic interactions. Thus, the antigen exposure is quite different as compared to that on the surface of microtitre wells, where phospholipids are coated in a layer of immobilized lamellar phospholipids [32]. Indeed, the present results obtained by TLC immunostaining showing the presence of aCL in more than half SN-APS patients (54.2%) confirm our previous reports [22] and suggest that this method may represent a useful tool to detect aCL, mainly in SN-APS patients. Moreover, all sera positive for anti-LBPA by TLC were also positive for CL. A possible limit of this method could be a relatively low sensitivity, since only 68% of "true" APS sera were positive for aCL by TLC.

Moreover, we have to consider that aPL represent a very heterogeneous family of antibodies because more than 30 different antibodies have been described in APS patients (the so-called autoantibody explosion in antiphospholipid syndrome) [33]. Among them, several "nonclassical" aPL are directed against platelets, glycoproteins, coagulation factors, lamins, mitochondrial antigens, and other cell surface markers [19]. With the aim to discover "new" antigenic targets of aPL, we identified, with a proteomic approach, vimentin/CL as a potential autoantigen in SN-APS patients [20]. In the present study we found serum antibodies against vimentin/CL in about 45% of SN-APS patients. Interestingly, these latter antibodies were detected in 4 SN-APS patients who were also negative for aCL by TLC immunostaining, supporting the view that antivimentin/CL test may be a relevant additional test for identification of aPL positive patients. Finally, we analyzed the SN-APS sera for the presence of antibodies against two major cofactor proteins for aPL, prothrombin and annexin V. Our results revealed that 3 out of 24 sera from SN-APS patients (12.5%) displayed antibodies to prothrombin and 1 (4.2%) to annexin V. The datum of antiprothrombin might be underestimated, since we tested antiprothrombin antibodies in the absence of phosphatidylserine. Indeed, anti-phosphatidylserine/prothrombin antibodies, which have been recently standardized and validated [34], seemed to represent a stronger risk factor for thrombosis than antiprothrombin [35]. However, interestingly, in SN-APS group, anti-prothrombin antibodies were detected in 3 patients, which were negative for aCL by TLC immunostaining.

Taken together, these findings indicate that the execution of all these tests (TLC immunostaining, antivimentin/CL, antiprothrombin, and antiannexin V) can be very useful for identification of autoantibodies in so-called SN-APS patients.

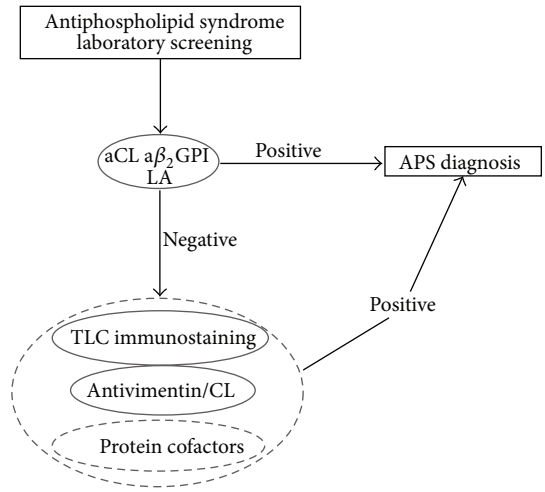

Figure 1: Proposed algorithm depicting the different diagnostic approaches in the most reliable sequence to reveal the hidden reactivity of the conventional aPL detection.

We can assert that, by using these approaches, it is possible to detect autoantibodies in the majority of the patients (79.2%). We suggest using all these different diagnostic approaches in the most reliable sequence, starting with TLC immunostaining, then antivimentin/CL ELISA, and eventually anticofactors ELISA to reveal the hidden reactivity of the conventional aPL detection (Figure 1). The combination of the first two diagnostic tests, TLC immunostaining and anti-vimentin/CL ELISA, detects aPL in about two-thirds of tested samples.

The presence of these antibodies has several implications. They might be used for early diagnosis of the syndrome, especially among patients at risk for thrombotic events and/or pregnancy morbidity. Moreover, autoantibody presence or absence might subclassify APS according to the association of these antibodies with clinical manifestations. At the end, they may help to identify patients who need secondary thromboprophylaxis with long-term anticoagulation, as well as prophylactic treatment during pregnancy.

However, although these approaches ameliorate our diagnostical possibilities, we are still unable to detect autoantibodies in a percentage of patients with a clinical picture suggestive of SN-APS. Other unidentified cofactors may be involved in sera reactivity. Further studies will shed light on "new" antigenic specificities in SN-APS as well as on the real importance of nonclassical antibodies found in SN-APS.

5. Conclusions

These findings suggest that in sera from patients with SN-APS, antibodies may be detected using "new" antigenic targets (mainly vimentin/cardiolipin) or methodological approaches different from traditional techniques (mainly TLC immunostaining). Thus, SN-APS represents a mosaic, in which antibodies against different antigenic targets may be detected.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Authors' Contribution

Fabrizio Conti and Antonella Capozzi equally contributed to this paper.

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Research Article

Anti-Nuclear Antibodies in Daily Clinical Practice: Prevalence in Primary, Secondary, and Tertiary Care

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For the diagnosis of systemic autoimmune rheumatic diseases (SARD), patients are screened for anti-nuclear antibodies (ANA). ANA, as assessed by indirect immunofluorescence (IIF), have a poor specificity. This hampers interpretation of positive results in clinical settings with low pretest probability of SARD. We hypothesized that the utility of positive ANA IIF results increases from primary to tertiary care. We retrospectively determined ANA, anti-ENA, and anti-dsDNA antibody prevalence in patient cohorts from primary (n = 1453), secondary (n = 1621), and tertiary (n = 1168) care settings. Results reveal that from primary care to tertiary care, ANA prevalence increases (6.2, 10.8, and 16.0%, resp.). Moreover, in primary care low titres (70% versus 51% and 52% in secondary and tertiary care, resp.) are more frequent and anti-ENA/dsDNA reactivities are less prevalent (21% versus 39% in secondary care). Typically, in tertiary care the prevalence of anti-ENA/dsDNA reactivities (21%) is lower than expected. From this descriptive study we conclude that positive ANA IIF results are more prone to false interpretation in clinical settings with low pretest probabilities for SARD, as in primary care. Whether alternative approaches, that is, immunoadsorption of anti-DFS70 antibodies or implementation of anti-ENA screen assays, perform better, needs to be determined.

1. Introduction

The hallmark of autoimmune diseases is the pathologic activity of the immune system of an organism directed against its own cells and tissues. The disease is a direct consequence of tissue and/or organ damage as mediated by autoreactive components of the immune system, that is, autoreactive T-lymphocytes and/or autoantibodies. For diagnostic purposes, autoantibodies are the most important analytes. Within the systemic autoimmune rheumatic diseases (SARD), anti-nuclear antibodies (ANA), directed against various cellular components, and associated autoantibodies, such as antibodies reactive with dsDNA and extractable nuclear antigens (ENA), are fundamental for diagnosis [1–3].

Traditionally, ANA are detected by indirect immunofluorescence (IIF) performed on human epithelial cells (HEp-2). This technique requires a multistage process consistent with visual determination of the staining pattern, serial titrations of positive sera, followed by a second test in which autoantigen specificity is determined [2, 4]. Recently, the American College of Rheumatology (ACR) stated that ANA detection by IIF is still considered the gold standard [5]. This was primarily based on the high sensitivity of the IIF assay and the inclusion of ANA detection by IIF assay in diagnostic criteria of systemic lupus erythematosus (SLE) and autoimmune hepatitis (AIH) [6–8]. In addition, ANA can also be considered as a screening test for samples that require further testing for autoantigen specificity, that is, dsDNA and ENA [2, 9].

The specificity of ANA detection by IIF, however, is relatively poor, especially when low titres are used for screening. Indeed, at a 1 : 40 serum dilution, 25–30% of healthy individuals may test positive for ANA and this increases even further upon ageing [1, 10]. Overall, it is recommended that the serum dilution that gives a specificity of 95% in healthy individuals should be used as cut-off [3]. Moreover, the

clinical significance rises with increasing titres [11, 12], as well as with the identification of the responsible autoantigen [1, 9]. Obviously, a positive ANA test must always be interpreted cautiously and only within the clinical context of the patient. In a clinical setting where the pretest probability of SARD is generally low, as in primary care, the added value of a positive ANA test is lower as compared to secondary and tertiary care situations where pretest probabilities of SARD are often higher [13].

In the current study, we determined the prevalence of ANA in primary (general practices), secondary (regional hospital), and tertiary care (university hospital). Besides data on ANA prevalence, also ANA titres and anti-ENA and anti-dsDNA antibodies were included in our analyses. We hypothesize that ANA prevalence, ANA titre, and anti-ENA/dsDNA reactivity increase from primary to tertiary care as these situations are expected to be also associated with an increasing pretest probability of SARD.

2. Materials and Methods

2.1. Patients/Participants. In the present study, three different patient populations from the southern part of The Netherlands were evaluated and compared with each other. These three populations consisted of patients who were tested for ANA between November 2011 and August 2012 in suspicion of an autoimmune disease. All ANA requests were considered to involve the diagnostic workup since none of the patients had requests for ANA (and/or anti-ENA/dsDNA) at least 4 years prior to the study period.

In the first patient population ($n = 1453$) ANA were requested by general practitioners (primary care). The second population ($n = 1621$) had an ANA request in a regional hospital (secondary care), while the third population ($n = 1168$) had an ANA request in a university hospital (tertiary care). Testing for ANA in the first and second cohorts was performed in the Atrium MC (Heerlen, The Netherlands), while the ANA tests in the third cohort were performed in the Maastricht University Medical Centre (MUMC, Maastricht, The Netherlands). Furthermore, in both regional and university hospitals, the origin, that is, hospital department, of ANA requests was documented.

2.2. Detection of ANA by IIF. ANA detection by IIF was performed on HEp-2000 cells according to the instructions provided by the manufacturer (Immuno Concepts, Sacramento, CA). Hep-2000 cells are transfected with the gene for SSA-60, which makes these cells more sensitive for SSA-antibody detection [14, 15]. Serum samples were screened in a 1 : 80 dilution. FITC-conjugated goat anti-human IgG antibody was used for detection of ANA. Five staining patterns were considered ANA positive: homogenous, atypical speckled, speckled, centromere, and nucleolar. In case of mixed-patterns, the pattern with the highest titre was included in the present study. Slides were evaluated with a fluorescent microscope (Axioskop, Carl Zeiss Microscopy GmbH, Jena, Germany) with LED light source. All slides were evaluated by two independent observers; in case of a difference in opinion, a third observer was decisive.

2.3. Detection of Anti-ENA Antibodies by LIA. The presence of anti-ENA antibodies was screened by a commercially available line immunoassay (ANA 3 Profile EUROLINE, Euroimmun, Lübeck, Germany). The assay was performed according to the manufacturer's instructions. The sera were diluted 1 : 100 in sample buffer. After the first incubation with diluted serum, a second incubation was performed with goat anti-human IgG linked to alkaline-phosphatase. Finally, a third incubation took place with bromochloroindolyl phosphate and nitro blue tetrazolium chloride (BCIP/NBT) as substrate to detect anti-ENA antibodies. Although the ANA 3 Profile EUROLINE kit enables detection of 15 different antigens, only eight were evaluated in the current study: Ro52, SS-A/Ro60, SS-B/La, nRNP/Sm, Sm, Scl-70 (topoisomerase 1), Jo-1, and CENP-B. Reading of the results was automated and the colour intensities of the reactions were evaluated by the EUROLineScan program (Euroimmun) to enable semiquantitative determination, that is, equivocal, 1+, 2+, and 3+. Results were considered positive for Sm, Scl-70, and Jo-1 if the intensity was at least equivocal, while for SS-A/Ro60, SS-B/La, nRNP/Sm, and CENP-B an intensity of at least 1+ was required. Finally, the cutoff for Ro52 was 2+.

2.4. Detection of Anti-ENA Antibodies by FEIA. Positive LIA results, as defined above, were confirmed with a commercially available FEIA (EliA, ImmunoDiagnostics, Thermo Fisher Scientific, Freiburg, Germany). This method uses highly purified (SmD) or recombinant (SS-A/Ro60, Ro52, SS-B/La, CENP-B, Scl-70, Jo-1, RNP70, and U1RNP) human antigens that are coated on irradiated polystyrene cups. The assay was performed according to the manufacturer's instructions. The sera were diluted 1 : 50 with dilution buffer. After binding of anti-ENA antibodies, the cups were washed and subsequently incubated with mouse anti-human IgG (heavy chain specific) conjugated to β-galactosidase. In case of antibody association, binding was detected fluorometrically using 4-methylumbellifery-β-D-galactoside (0.01%) as substrate. All assay procedures were fully automated in an ImmunoCAP250 (Thermo Fisher Scientific). The reference range was supplied by the manufacturer. For all antigens, values above 10 U/mL were considered positive.

2.5. Detection of Anti-dsDNA Antibodies by FEIA and CLIFT. In primary and secondary care, anti-dsDNA antibodies were detected with a commercially available FEIA (EliA, ImmunoDiagnostics, Thermo Fisher Scientific). This method uses a circular plasmid dsDNA, purified from *Escherichia coli*, as antigen. The assay was performed according to the manufacturer's instructions and as described for the anti-ENA antibodies. The sera were diluted 1 : 10 in dilution buffer and values above 15 U/mL were considered positive.

In tertiary care, anti-dsDNA antibodies were detected by the *Crithidia luciliae* immunofluorescence test (CLIFT; Immuno Concepts). Serum samples were screened in a 1 : 10 dilution. FITC-conjugated goat anti-human IgG antibody

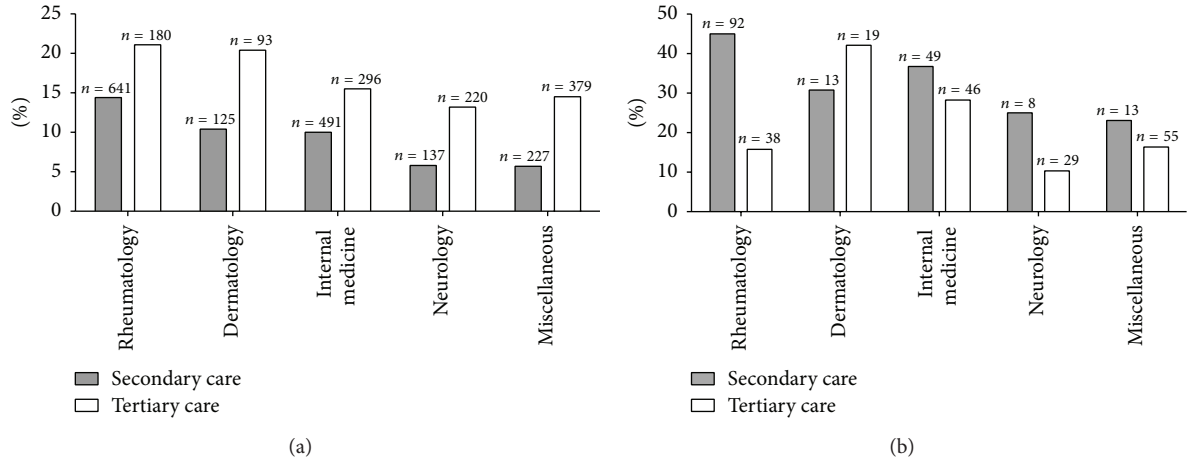

Figure 1: Relative presence of ANA and anti-ENA/dsDNA antibodies stratified for requesting clinical department. (a) The prevalence (%) of ANA positive sera in the rheumatology, dermatology, internal medicine, neurology, and miscellaneous departments of secondary (grey bars) and tertiary care (white bars) is displayed relative to the total amount of ANA requests per department (*n* above bars). (b) The prevalence (%) of anti-ENA/dsDNA reactivity in ANA positive sera in secondary (grey bars) and tertiary care (white bars) is displayed relative to the total amount of ANA positive sera (*n* above bars).

was used for detection of anti-dsDNA antibodies. Slides were evaluated with a fluorescent microscope (Axioskop, Carl Zeiss Microscopy GmbH) with LED light source. All slides were evaluated by two independent observers; in case of a difference in opinion, a third observer was decisive.

2.6. ANA/ENA Algorithm. All samples were tested first by ANA IIF. If the result was negative, no further testing was performed, unless specifically requested. However, the results of these additional tests were not included in the present study. If the ANA IIF was positive, irrespective of the staining pattern, titration was performed (1 : 320 and 1 : 1280). If a homogenous ANA pattern was detected, testing for anti-dsDNA antibodies was performed by FEIA (primary and secondary care) or CLIFT (tertiary care) [16]. Additionally, in case of a homogenous, (atypical) speckled, or centromere pattern, typing for anti-ENA antibodies was performed by LIA. Positive LIA results, as defined above, were tested by FEIA for confirmation. Confirmation was achieved if FEIA results were unequivocal positive. Because of the high correlation between the atypical speckled ANA pattern and SS-A/Ro60 antibodies [2], these antibodies were considered positive if the atypical speckled ANA pattern was observed in combination with either a positive LIA or a positive FEIA. Similarly, anti-CENP-B antibodies were considered positive when a centromere ANA pattern was observed in combination with a positive anti-CENP-B result in at least one of both anti-ENA antibody test systems. Since the LIA does not enable specific distinction of anti-RNP antibodies, positivity for the nRNP/Sm complex was followed by testing for antibodies against RNP70 and U-RNP by FEIA. Positivity of any of these two entities was interpreted as RNP positive. The same algorithm was applied to all requests within the 3 cohorts.

2.7. Statistical Analyses. All data analyses were performed using SPSS version 19.0 (SPSS Inc., Chicago, IL) or Graphpad Prism version 6 (Graphpad Software Inc., La Jolla, CA).

The Kolmogorov-Smirnov analysis was performed to determine whether the age distributions of the three study populations were normally distributed. Furthermore, the Chi square test, with Yates' correction if appropriate, was performed when comparing proportions of groups. In the case of small samples, Fisher's exact test was performed instead. A P value of <0.05 was considered statistically significant.

3. Results

3.1. Study Population and Origin of ANA Requests. Of the three patient populations included, 90 (6.2%), 175 (10.8%), and 187 (16.0%) patients from primary ($n = 1453$), secondary ($n = 1621$), and tertiary care ($n = 1168$), respectively, were tested positive for ANA and were therefore eligible for the current study. Gender (F/M) and age (median and range) distribution were as follows: 78/12 and 57.2 (15–95) for primary care, 129/46 and 57.0 (17–93) for secondary care, and 130/57 and 57.3 (3–84) for tertiary care.

The gender distribution differed significantly ($P = 0.009$) due to a strong female preponderance in primary care. The age distribution differed significantly ($P = 0.005$) due to the fact that in tertiary care age distribution was skewed negatively.

Evaluation of the origin, that is, hospital departments, of the ANA requests in secondary and tertiary care revealed four departments, that is, rheumatology, dermatology, internal medicine, and neurology, which requested the majority of the ANA screening tests (Figure 1(a)), that is, 86% and 68%,

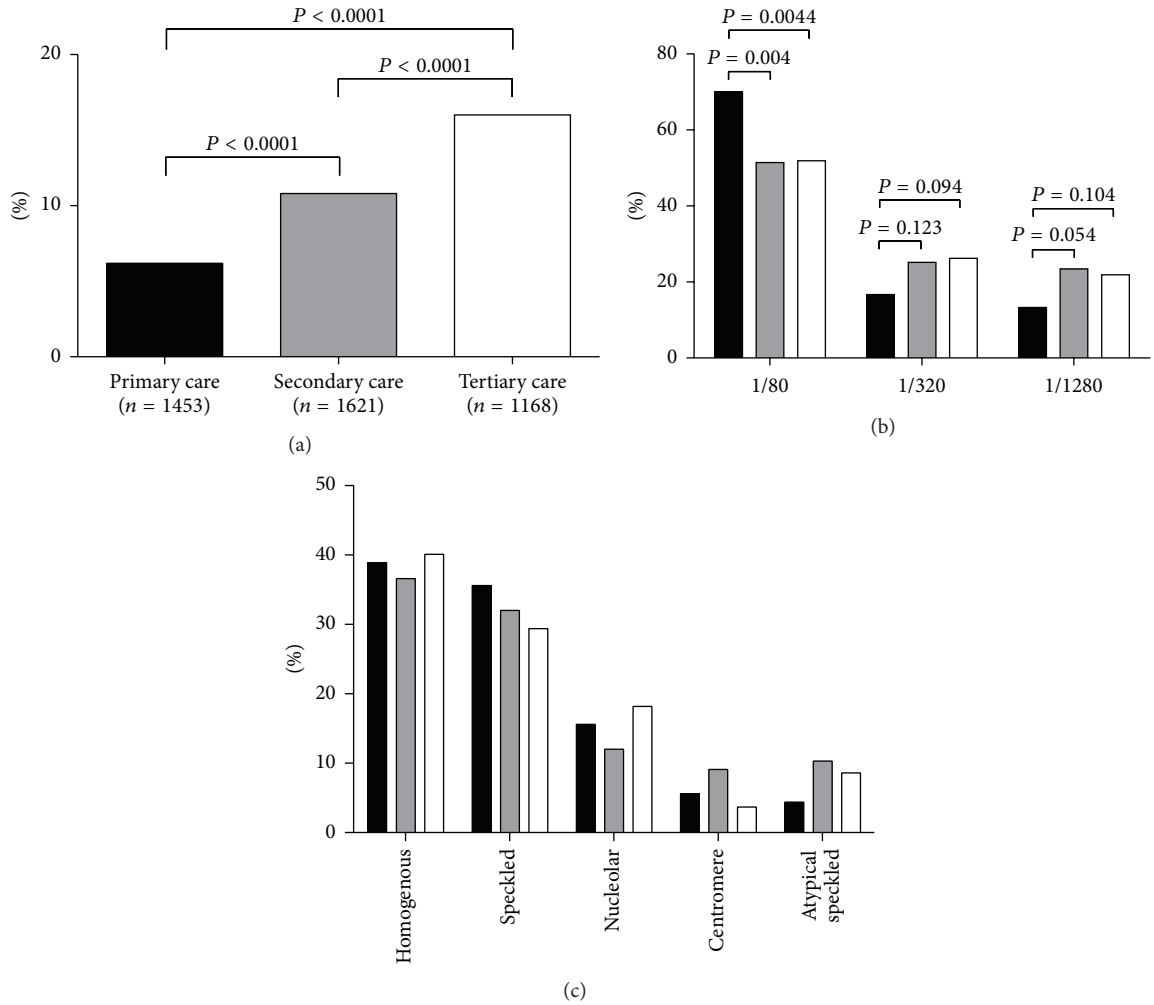

Figure 2: ANA reactivity stratified for primary, secondary, and tertiary care. The relative prevalence (%) of ANA positive sera is presented for primary, secondary, and tertiary care (a). The relative prevalence (%) of ANA titres (b) and patterns (c) is displayed in primary (black bars), secondary (grey bars), and tertiary care (white bars).

respectively. All other departments were collectively grouped as miscellaneous. This group was most diverse in tertiary care.

3.2. ANA Prevalence and Titre Increase from Primary to Tertiary Care. The prevalence of ANA in the 3 distinct clinical settings is depicted in Figure 2(a). The relative increase in the prevalence from primary to tertiary care is statistically significant. The higher relative ANA prevalence in tertiary care (16.0%) versus secondary care (10.8%) was apparent in all 4 clinical disciplines that requested the majority of ANA screening test (Figure 1(a)); this also holds for the other clinical disciplines (data only shown as pooled results). Typically, in secondary care 39.5% of overall ANA requests came from the rheumatology department, while in tertiary care this was 15.4%.

Within the positive ANA cohorts, patients from primary care had relatively low titres as compared to secondary and tertiary care (Figure 2(b)). Indeed, out of 90 positive ANA tests 63 sera (70%) revealed an ANA titre of 1 : 80. In secondary and tertiary care, a titre of 1 : 80 was obtained in 51.4% and 51.9% of the patients, respectively. Consequently, higher titres were observed more frequently in secondary and tertiary care than in primary care. The distribution in titres between secondary and tertiary care was not different.

At first glance, there is no apparent difference in the distribution of ANA patterns between the three health care levels (Figure 2(c)). Also, comparison of the distribution of ANA patterns with a titre of 1 : 1280, considered to have the highest positive likelihood ratio, showed no significant differences (data not shown).

3.3. Anti-ENA and Anti-dsDNA Antibodies Prevalence Is Highest in Secondary Care. The prevalence of anti-ENA and anti-dsDNA antibodies, as defined by the algorithm described above, is presented in Figure 3(a). In the primary care, 19 (21.1%) patients with positive ANA ($n = 90$) were tested positive for anti-ENA and/or anti-dsDNA antibodies, that is, 1.3% of the total cohort. In the secondary and tertiary care, 68 of 175 (38.9%) and 40 of 187 (21.4%) ANA positive patients revealed anti-ENA and/or anti-dsDNA reactivity,

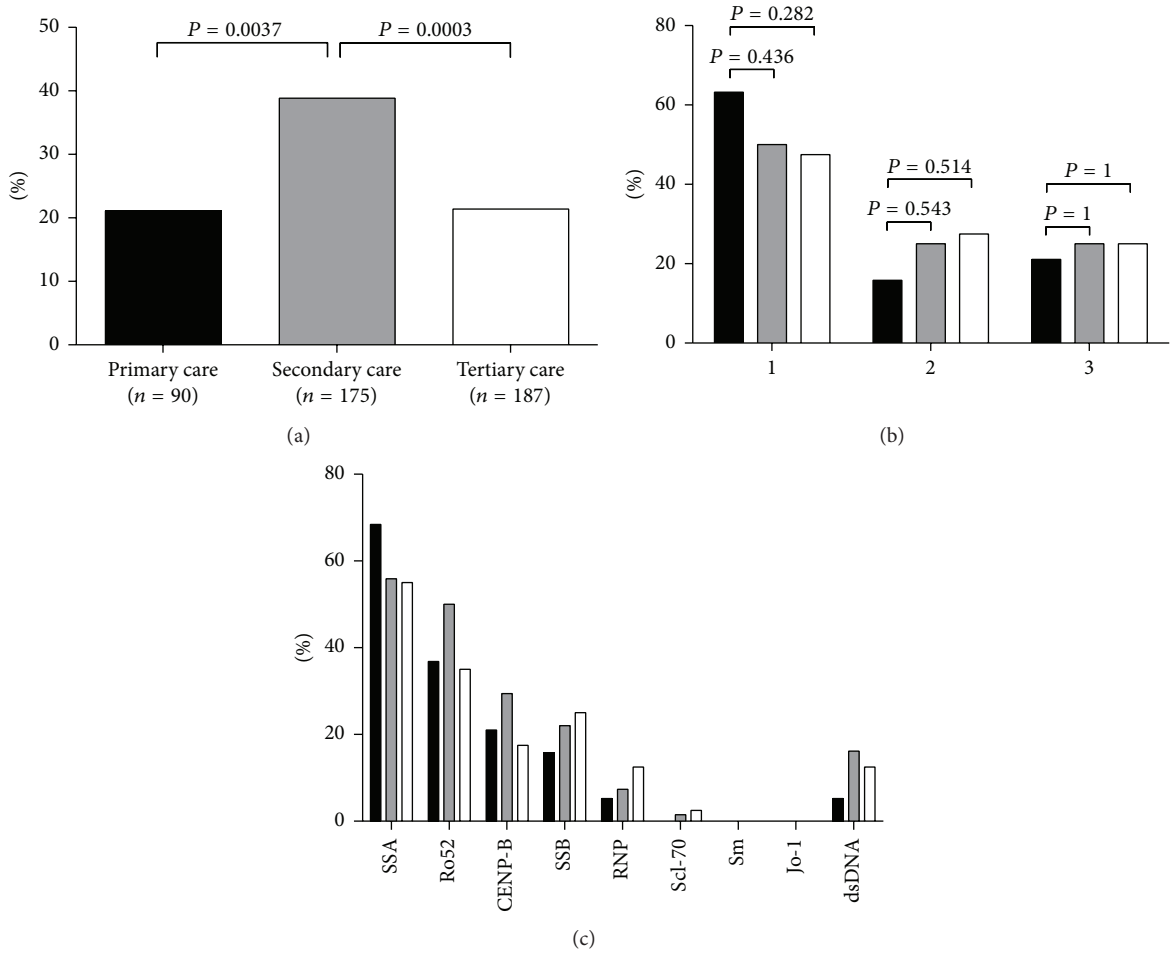

Figure 3: Anti-ENA/dsDNA reactivity stratified for primary, secondary, and tertiary care. The relative prevalence (%) of anti-ENA/dsDNA reactivity in ANA positive sera is presented for primary, secondary, and tertiary care (a). The relative prevalence (%) of 1 or more specificities (b) and type of specificities (c) is displayed in primary (black bars), secondary (grey bars), and tertiary care (white bars). Notably, in primary and secondary care anti-dsDNA antibodies were detected by FEIA, while in tertiary care CLIFT was the method of choice.

respectively. This is 4.2% and 3.4% of the total secondary and tertiary care cohorts, respectively ($P = 0.367$). Significantly more positive anti-ENA and/or anti-dsDNA results were found in the total secondary and tertiary care cohorts than in the primary care cohort ($P < 0.0001$ and $P = 0.0006$, resp.). In secondary care, the prevalence of anti-ENA and/or anti-dsDNA reactivity within the positive ANA samples was the highest in the requests from rheumatology (Figure 1(b)). In tertiary care, however, departments of rheumatology and neurology had, on average, reduced prevalence of anti-ENA and/or anti-dsDNA reactivity as compared to the departments of dermatology and internal medicine (Figure 1(b)).

Since the relevance of anti-ENA and anti-dsDNA antibodies is considered to increase when combined reactivity is observed, we analysed the prevalence of anti-ENA and/or anti-dsDNA antibodies in relation to combined reactivity (Figure 3(b)). In the primary care setting, single positivity appeared to be more abundant than in secondary and tertiary care, but this difference was statistically not significant. No differences were observed between secondary and tertiary care settings either. With respect to the antigens recognized by the specific antibodies, no apparent differences were observed in the prevalence of antibodies reactive to RNP, SSA60, Ro52, SSB, CENP-B, and dsDNA (Figure 3(c)). Antibodies reactive to Sm and Jo-1 were not detected in any sample. Anti-Scl70 antibodies were only detected in samples of patients from secondary and tertiary care. However, the absolute number, one in each cohort, was low in both of these settings.

4. Discussion

In the present study on the analyses of ANA prevalence, ANA titre and anti-ENA specificity in the primary, secondary, and tertiary care, our results indicate that (i) ANA prevalence significantly increases from primary to tertiary care, (ii) low titres (1 : 80) are more frequently observed in the primary care, and (iii) anti-ENA and anti-dsDNA specificities are significantly more prevalent in the secondary care than in the primary care. Typically, the latter observation does not hold for the tertiary care.

Interpretation of the data obtained in the current study is highly dependent on the viewpoint on clinical utility of an ANA test result. It is tempting to start from the clinical utility of a positive ANA result. This result may help the clinician to identify a patient with SARD, but especially in situations with low pretest probabilities of such diseases, the risk of false interpretation of a positive ANA is high. This risk of false positive interpretation will decrease if the positive ANA is characterized by high titre and includes (multi)-reactivity for ENA and/or dsDNA, since these characteristics are associated with higher positive likelihood ratios [11, 12, 17]. Next, one has to realize that ANA testing is performed in the context of multiple diseases, varying from distinct SARD to autoimmune liver diseases. Interpretation of a positive ANA test may be different for each distinct disease: patients with, for instance, AIH or systemic sclerosis often have a positive ANA with no ENA reactivity, while a positive ANA test as such is part of the classification criteria for SLE, as well as AIH [6–8]. A negative ANA test result, on the other hand, may also be very useful to exclude a specific set of diseases and may drive attention to other diseases. Again, this differs for the distinct SARD: a negative ANA test has a high negative predictive value for SLE and systemic sclerosis but is less helpful to exclude Sjögren's syndrome or myositis [2, 3]. Obviously, definite interpretation of our dataset is hampered by the lack of clinical data from the patients of the three cohorts. The assumption of the primary care having a relatively low pretest probability was based on previous studies [13, 18, 19]. For future studies, we recommend the inclusion of clinical data in order to be able to thoroughly assess pretest probabilities and strengthen our assumptions. This study, however, also has noteworthy strengths, that is, only data obtained during the diagnostic workup of patients (not follow-up samples) were included and the same testing algorithm and reagents (except for detection of anti-dsDNA antibodies) were used for all three patient cohorts.

As expected, we observed a gradual increase in the prevalence of ANA from primary to tertiary care. In our present study ANA was detected by IIF in a screening dilution of 1 : 80. In several studies it has been reported that 10–15% of healthy controls are ANA positive in this serum dilution [1, 10, 20]. Obviously, a positive ANA test result is not only dependent on the dilution factor but also on the quality of other reagents, that is, fluorescent conjugate, cell substrate, and the fluorescent microscope [4]. For standardization purposes, it has, therefore, been recommended to screen for ANA with a specificity of 95% [3]. Taking this into account, the mere presence of ANA in our primary care cohort (prevalence 6.2%) lacks discriminating power to identify patients with SARD. On the other hand, due to the high negative predictive value of a negative ANA result, the general practitioner can reliably exclude certain diseases. Furthermore, since both high titre and anti-ENA and/or anti-dsDNA (multi-)reactivity may increase the likelihood of identifying a patient with SARD [11, 12, 17], it is apparent in our primary care cohort that 30% of ANA positive sera are of medium to high titre ($n = 27$), 21% contain anti-ENA and/or anti-dsDNA reactivity ($n = 19$), and 37% of the latter reveal multireactivity ($n = 7$). In our secondary and tertiary cohorts, the ANA prevalence is also relatively low (11 and 16%, resp.), but in both clinical settings about half of the ANA positive sera are of high titre (48-49%). As expected, the observed anti-ENA and/or anti-dsDNA reactivity of ANA positive sera in the secondary care (39%) is higher than in the primary care. Surprisingly, anti-ENA and/or anti-dsDNA reactivity in the tertiary care (21%) is lower than in the secondary care and similar to the primary care. This might be related to the spectrum of diseases investigated in the tertiary versus the secondary centres. Also the higher number of ANA requests by tertiary care departments not typically involved in the diagnosis of SARD might be the result of academic profiling of the respective departments. For instance, the cardiology department of the MUMC is specialized in inflammatory cardiomyopathies and a possible autoimmune aetiology of these diseases. Cardiology requested 108 ANA tests (9.2%), revealing 13% ANA positive results of which 20% was anti-ENA and/or anti-dsDNA antibody positive. A second important difference is the presence of a division of clinical immunology within the department of internal medicine of the tertiary care hospital, implying that many patients with SARD are evaluated by clinical immunologists instead of rheumatologists. The latter difference might at least explain the lower relative prevalence of anti-ENA and anti-dsDNA antibodies in the rheumatology department of the tertiary care hospital.

Our study shows that in secondary care the majority of positive ANA results (92.6%), as well as the positive anti-ENA and anti-dsDNA results (95.6%), are linked to the departments of rheumatology, dermatology, internal medicine, and neurology. Obviously, patients with the highest pretest probabilities for SARD, as associated with the initial clinical presentation, are most likely referred to these clinical departments. In clinical settings with lower pretest probabilities, ANA positive sera are more likely to be of no clinical significance [13, 18, 19]. It might therefore be an option, in particular in primary care settings, to move away from the traditional ANA screening test. In this context, the recent discovery of the dense fine speckled (DFS70) antigen is promising and could offer a possible solution for the identification and exclusion of positive sera with no clinical relevance [21, 22]. The typical ANA dense fine speckled pattern (DFS), known to be associated with the DFS70 antigen, has been found to be commonly prevalent in healthy individuals with ANA positive sera (33.1%), whereas in SARD patients 0.0% of the sera revealed a DFS pattern [22]. Another study revealed that 2-3% of SARD patients had antibodies directed to the DFS70 antigen [23]. Obviously, in SARD patients other anti-ENA antibodies might be present that hamper correct recognition of the DFS IIF pattern. Indeed, the identification and correct interpretation of the DFS pattern might prove to be challenging for diagnostic laboratories and would require additional training [24]. The laboratories involved in the current study did not distinguish the DFS pattern. Moreover, the majority of patterns recognized do not seem to be compatible with a DFS pattern, but this might be the consequence of only interpreting the strongest pattern in the current study. Since the identification of the DFS pattern might be challenging for routine diagnostic laboratories and

inaccurate interpretation can have significant consequences [25], an immunoadsorption protocol to diminish anti-DFS70 antibody reactivity to HEp-2000 cells could be implemented in the current IIF assay in order to significantly improve the performance characteristics of the ANA IIF test [21, 25]. This approach sustains recognition of SARD-related autoantibodies in sera with combined reactivities, that is, anti-DFS70 and other anti-ENA antibodies. Another alternative testing algorithm could, instead of ANA IIF, include solid phase assays including multiplex and screening assays for well-defined anti-ENA and anti-dsDNA reactive antibodies [11, 12, 26, 27]. Both approaches enable to distinguish or reduce the number of positive ANA results lacking clinical relevance. In addition, multiplex anti-ENA screening assays are considered to better recognize particular antigens, for example, SSA and Jo-1, as compared to ANA IIF testing [3, 11, 12, 28].

Altogether, the results indicate that in the primary care the usage of traditional ANA screening tests is more prone to false-interpretation of positive ANA results. Rather, an alternative testing algorithm for detection of patients with SARD might be more appropriate. This might either be achieved by immunoadsorption of anti-DFS70 antibodies or direct screening for anti-ENA antibodies. Obviously, a patient with severe clinical manifestations typical for SARD presenting in a primary care setting should be referred directly, that is, without any laboratory testing, to the rheumatologist or clinical immunologist. This recommendation may not only apply for general practitioners but may also hold for clinical departments that are less likely to encounter patients suspected of SARD.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Analysis of Autoantibodies to 3-Hydroxy-3-methylglutaryl-coenzyme A Reductase Using Different Technologies

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Diagnostic tests are needed to aid in the diagnosis of necrotizing myopathies associated with statin use. This study aimed to compare different technologies for the detection of anti-HMGCR antibodies and analyze the clinical phenotype and autoantibody profile of the patients. Twenty samples from myositis patients positive for anti-HMGCR antibodies using a research addressable laser bead assay and 20 negative controls were tested for autoantibodies to HMGCR: QUANTA Lite HMGCR ELISA and QUANTA Flash HMGCR CIA. All patients were also tested for antibodies to extractable nuclear antigens and myositis related antibodies. To verify the specificity of the ELISA, 824 controls were tested. All three assays showed qualitative agreements of 100% and levels of anti-HMGCR antibodies showed significant correlation: Spearman's rho > 0.8. The mean age of the anti-HMGCR antibody positive patients was 54.4 years, 16/20 were females, and 18/20 had necrotizing myopathy (two patients were not diagnosed). Nine out of 20 anti-HMGCR positive patients were on statin. All patients with anti-HMGCR antibodies were negative for all other autoantibodies tested. Testing various controls showed high specificity (99.3%). Anti-HMGCR antibodies are not always associated with the use of statin and appear to be the exclusive autoantibody specificity in patients with statin associated myopathies.

1. Introduction

Autoantibodies are a hallmark in the diagnosis of many systemic autoimmune rheumatic diseases (SARD) including idiopathic inflammatory myopathies (IIM) (reviewed in [1, 2]). Most of those autoantibodies are directed to intracellular proteins, including nuclear and cytoplasmic antigens, and based on their specificity, autoantibodies in IIM can be grouped into myositis specific autoantibodies (MSA) and myositis associated autoantibodies (MAA) (reviewed in [1–3]). The presence of MSA and MAA has become a key feature for classification and diagnosis of IIM and they are increasingly used to define clinically distinguishable IIM subsets. Among the MSA, autoantibodies against aminoacyl-tRNA synthetases (ARS) were detected in 25–35% of IIM patients. Other autoantibodies in IIM are directed to the signal recognition particle (SRP), chromodomain helicase DNA binding protein 4 (Mi-2), SAE/small ubiquitin-related modifier (SUMO-1), MJ/nuclear matrix protein 2 (NXP2), melanoma differentiation-associated gene 5 (MDA5)/clinically amyopathic dermatomyositis p140 (CADM-140), and transcription

intermediary factor (TIF1-) gamma (p155/140) [2]. Anti-Jo-1 antibodies are the most common, predominantly found in 15–30% of patients with polymyositis (PM) and in 60–70% of those with interstitial lung disease (ILD). Autoantibodies directed towards other ARS are less common, each reaching less than 5% prevalence in IIM. MSA and MAA are commonly detected using immunoprecipitation (IP) or line immunoassays (LIA) [4]. Muscle pain and weakness are common side effects of statins which are commonly used to reduce cholesterol levels. About 5% of statin users experience muscle pain and weakness during statin treatment. In 2010, antibodies to 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMGCR) have been identified in patients with autoimmune necrotizing myopathies associated with statin use [5–7]. Recently, a significant difference between statin-exposed and statin-unexposed anti-HMGCR positive patients has been found [8]. Therefore, diagnostic tests are needed to aid in the diagnosis of this severe clinical condition [9, 10]. This study aimed to compare different technologies for the detection of anti-HMGCR antibodies and analyze the clinical phenotype and autoantibody profile of the patients and to investigate the epitope specificity of anti-HMGCR antibodies.

2. Materials and Methods

2.1. Sera. A total of 20 samples from myositis patients positive for anti-HMGCR antibodies (see Table 1) using a research addressable laser bead assay (ALBIA, Rouen, France) identified in a previous study [11] and 20 negative controls (age and sex matched) were collected and tested using various methods. To verify the specificity of the QUANTA Lite HMGCR ELISA a total of 824 controls were tested (for details see Section 3). Diagnoses of the patients were established based on the respective disease classification criteria and as previously described [12].

Collection of patient samples was carried out according to local ethics committee regulations. Patient data was anonymously used in keeping with the latest version of the Helsinki Declaration of human research ethics.

2.2. HMGCR Antigens and Western Blotting. Two different HMGCR antigens were used in the present study. The first antigen was obtained from a commercial source (Sigma) and consists of the HMG-CoA reductase catalytic domain expressed in *E. coli* and fused to GST protein with a final molecular weight of 76 kDa (including the fusion protein). The other antigen was developed at INOVA Diagnostics as follows. The HMGCR DNA was cloned into the pIEx/Bac-3 vector using Homo sapiens HMGCR and transcript variant 1 (NM_000859.2) amino acids 427–888. The clone is N-terminal 10X Histidine tagged and expressed in Sf9 cells with a molecular weight of 51 kDa. The cells were grown to 2–4 $\times 10^6$ cells/mL in sf900 II SFM medium and infected using 20 mL of HMGCR baculovirus per 1 L of cell culture. They were incubated at 27°C for 108 hrs while rotating at 140 rpm. The cells were harvested by centrifuging at 8000 rpm for 15 minutes using an SLC-6000 rotor. The cell pellets were washed with PBS, centrifuged at 4000 rpm and the pellets were stored at −80°C prior to extraction.

The HMGCR antigen was extracted using 1 M NaCl, 20 mM Tris, 0.25% CHAPS, and 10 mM Imidazole buffer, pH 8.0. Protease inhibitor tablets were added and the cells sonicated for 3 minutes. The sonicated mixture was centrifuged at 30,000 rpm for 30 minutes using a 50.2Ti rotor. The supernatant was collected and run over a Ni++ NTA IMAC column equilibrated on the extraction buffer. The column was washed using 1 M NaCl, 20 mM Tris, 0.25% CHAPS, and 140 mM Imidazole buffer, pH 8.0 and eluted using 1 M NaCl, 20 mM Tris, 0.25% CHAPS, and 400 mM Imidazole buffer, pH 8.0. The elution was collected and buffer exchanged using a G25 SEC equilibrated using 1 M NaCl, 10 mM Tris, 0.25% CHAPS, and 0.09% NaN3 buffer, pH 8.0. The HMGCR antigen was quantified using the calculated extinction coefficient and A280 absorbance measured using a UV/Visible spectrophotometer and stored at −80°C.

In-house antigen (lot #ALO38) was compared to Sigma antigen (HMG-CoA reductase H7039 059K4055) via SDS PAGE/western blot. Both antigens were loaded to a 15-well 4–12% Bis-Tris prepact polyacrylamide gel (Life Technologies, Carlsbad, California), at 0.5 μg per well. A SeeBlue Plus2 prestained MES ladder (Life Technologies) was run in lane 1 for molecular weight determination. Electrophoresis was performed using a Mini Blot gel box and MES running buffer (Life Technologies). Proteins were run at 200 volts for 45 minutes using a BioRad Model 200/2.0 power supply.

The ladder in lane 1 was cut from the gel to be stained separately. The remaining samples were then transferred to a nitrocellulose membrane using a life technologies iBlot transfer unit. The nitrocellulose membrane was rinsed in DI water then allowed to dry. The membrane was then cut into 8 strips with each containing 1 lane of each antigen.

Strips were then incubated in HRP Sample Diluent (INOVA 508551) for 30mins followed by incubation with the appropriate patient samples at a 1 : 100 dilution for 1 hr. The strips were then washed with HRP Wash (INOVA 508552) 4 × 5 min and incubated with a goat anti-human secondary antibody diluted 1 : 3000 (Jackson Immuno Research) in HRP sample diluent for 1 hr. Strips were washed 4 × 5 min in DI water then developed with BCIP/NBT (Moss, Inc.).

2.3. Diagnostic Tests

2.3.1. Assays for Anti-HMGCR Antibodies. The QUANTA Flash HMGCR (research use only) assay is a novel CIA that is currently used for research purposes only and utilizes the BIO-FLASH instrument (Biokit s.a., Barcelona, Spain), fitted with a luminometer, as well as all the hardware and liquid handling accessories necessary to fully automate the assay [13].

The QUANTA Flash assay for this study was developed using recombinant human HMGCR antigen coupled to paramagnetic beads. Prior to use, the reagent pack containing all the necessary assay reagents is gently inverted thirty times and the sealed reagent tubes are then pierced with the reagent pack lid. Patient serum samples are prediluted

Table 1: Clinical and serological data of anti-HMGCR positive sera.

ID	ALBIA (>20)	CIA (>10,000)	ELISA (>20)	Gender	Age	Statin	ADO (ys)	CK* (25–160 U/L)	Necrosis
3	928	201891	149.2	F	62	+	59	1000	+
5	496	213681	113.5	F	53	−	52	13777	+
6	960	259814	193.1	M	59	−	47	8500	+
9	1008	291111	184.9	F	22	−	18	ND	+
10	784	138696	284.3	F	16	−	13	1500	+
11	512	244281	381.8	F	66	+	60	6500	+
13	768	179810	153.5	F	69	+	63	492	Very few fiber
15	320	184190	174.7	F	44	−	21	ND	+
17	800	254442	139.4	F	60	−	54	6000	+
18	1136	218777	126.3	F	53	+	48	4106	+
26	280	119734	57.9	M	67	+	66	5700	+
27	576	180528	88.9	F	17	−	12	17000	+
28	528	288198	93.8	M	65	−	52	500	+
29	84	79904	29.8	F	75	+	73	7429	No biopsy
30	196	135149	49.7	F	72	+	68	3700	+
31	608	250484	324.1	F	35	−	26	5700	+
32	424	141861	932.1	M	81	−	82	ND	+
34	400	208075	118.5	F	84	+	83	4119	ND
37	212	160034	83.3	F	20	−	15	612	+
39	142	115671	56.6	F	67	+	58	2000	−

CK: creatinine kinase; CIA: chemiluminescence immunoassay; ALBIA: addressable laser bead assay; ADO: age at disease onset; ND: not determined.
*Not all CK values are derived from the time point when serum was obtained for anti-HMGCR testing.

with sample buffer in small disposable plastic cuvettes. Small amounts of the diluted patient serum, the beads, and the assay buffer are all combined into a second cuvette, mixed, and then incubated for 9.5 minutes at 37°C. The magnetized beads are sedimented using a strong magnet in the washing station and washed several times followed by addition of isoluminol conjugated anti-human IgG and again incubated for 9.5 minutes at 37°C. The magnetized beads are sedimented and washed repeatedly. The isoluminol conjugate is oxidized when sodium hydroxide solution and peroxide solutions ("Triggers") are added to the cuvette, and the flash of light produced from this reaction is measured as Relative Light Units (RLUs) by the BIO-FLASH optical system. The RLUs are proportional to the amount of isoluminol conjugate that is bound to the human IgG, which is in turn proportional to the amount of anti-HMGCR antibodies bound to the antigen on the beads.

2.3.2. QUANTA Lite HMGCR. ELISA plates coated with recombinant HMGCR were incubated with diluted patient samples. Assay procedure followed standard protocol of QUANTA Lite assays (INOVA Diagnostics). A five-point calibration curve was used to convert optical density values into units. The cut-off was defined as the 99% percentile of level in a previous internal study based on disease controls.

2.3.3. Quantitative Addressable Laser Bead Immunoassay (ALBIA). The titration of anti-HMGCR antibodies was performed using a Luminex-based immunoassay, as described elsewhere [14]. Briefly, the recombinant human HMGCR catalytic domain was coupled to fluorescent BioPlex COOH-microspheres (Biorad, Hercules, CA) with the BioPlex amine coupling kit according to manufacturer's protocol. A 10 μL volume containing 1,250 beads was incubated with patient's serum, in 96-well plates for 2 h. Beads were collected by filtration and washed before adding biotinylated mouse anti-human IgG Ab. After 1 h incubation and washing, anti-HMGCR autoantibodies were detected using streptavidin-R-phycoerythrin. Anti-HMGCR Ab titers were calculated from the Mean Fluorescent Intensity by comparison with a calibrator consisting in a human positive serum whose titer was arbitrarily set to 100 Arbitrary Units (A.U/mL). The threshold of positivity of this assay is 20 AU/mL.

2.4. Other Assays. All patients were also tested using assays for the detection of antibodies to extractable nuclear antigens (ENA, BMD, and Thermo Fisher) and myositis related antibodies (Scleroderma and myositis profile, D-Tek, Belgium).

2.5. Indirect Immunofluorescence on HEp-2 Slides. Autoantibodies were detected by indirect immunofluorescence on HEp-2000 cells (Reference SA2014-Ro, Immunoconcepts, Sacramento, CA, USA). Sera were tested at 1/80 screening dilution in PBS buffer, using a FITC-coupled antibody against human IgG (h + l). On these cells, the fluorescence pattern

suggestive for anti-HMGCR antibodies is a finely granular cytoplasmic staining on a minority (3% or less) of cells with perinuclear reinforcement.

2.6. Epitope Mapping Studies. Autoantibodies to various peptides were studied using PEPperCHIP technology (PEPperPRINT GmbH, Heidelberg, Germany) [15, 16]. Peptide arrays were blocked using blocking buffer (Rockland blocking buffer MB-070 (60 min before the first assay). Sera were diluted 1 : 1000 in incubation buffer (PBS, pH 7.4 with 0.05% Tween 20 and 10% Rockland blocking buffer) and incubated for 16 h at 4°C and shaking at 500 rpm. Arrays were then washed (2 × 1 min after each assay with Washing Buffer (PBS, pH 7.4 with 0.05% Tween 20). Secondary antibody (F(ab')2 goat anti-human IgG (H + L) DyLight680) diluted 1 : 5000 was added and incubated 30 min. Identified epitopes were synthesized as soluble peptides, coated to ELISA plates and tested with anti-HMGCR positive samples. By comparing the PEPperPRINT sequence reactivity data to public domain structures of HMGCR the reactive sequences that were likely to be accessible to antibodies were determined to be ^{531}GYMPIPVGVAGPL^{543}, 748GYNAHAANIVTAI^{760}, ^{554}MATTEGCLVASTN^{566}, ^{689}TDKKPAAINWIEG^{701}, ^{561}CLVASTNRGCRAI^{573}, and ^{702}RGKSVVCEAVIPA^{714}. Peptides were synthesized by BioSynthesis (San Diego, CA) as biotinylated constructs and tested via streptavidin ELISA assay.

2.7. Statistical Evaluation. The data was statistically evaluated using the Analyse-it software (Version 1.62; Analyse-it Software, Ltd., Leeds, UK). *Spearman's correlation* and *Cohen's kappa agreement test* were carried out to analyze the agreement between portions. P values < 0.05 were considered significant.

3. Results

3.1. Comparison of Different Antigens and Different Methods for the Detection of Anti-HMGCR Antibodies. As the first step, the INOVA HMGCR antigen was compared to the Sigma antigen using western blot analysis to analyze the size, purity, and the reactivity pattern of both antigens. The western blot shows one anti-HMGCR positive sample for both INOVA and Sigma antigens. Using the Sigma antigen, several bands are stained by the serum (highest band ~ 75 kDa). In contrast, using the INOVA antigen, only one distinct band is recognized and stained (~55 kDa). Next, the antigens were compared by ELISA and the results obtained with the 40 samples were highly correlated (ρ = 0.80, see Figure 1). Subsequently, anti-HMGCR antibodies were detected using ALBIA, ELISA, and CIA and all three assays showed qualitative agreements of 100% (see Figure 2). In addition, the levels of anti-HMGCR antibodies also showed significant correlation: ELISA versus ALBIA, ρ = 0.84 (95% confidence interval, 0.72–0.91), ALBIA versus CIA, ρ = 0.89 (95% CI, 0.80–0.94), and ELISA versus CIA, ρ = 0.86 (95% CI, 0.75–0.92).

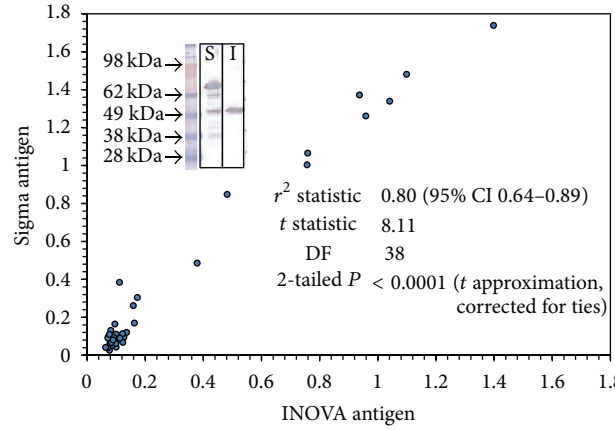


Figure 1: Comparison of two different antigens for the detection of anti-HMGCR antibodies using western blot and ELISA. The western blot shows the staining of a serum with anti-HMGCR antibodies of INOVA (I) and Sigma (S) antigen. A total of 40 samples with or without anti-HMGCR antibodies were compared by ELISA and the Spearman correlation shows good correlation between the two antigens.

3.2. Characteristics of Patients with Anti-HMGCR Antibodies and Serological Profiling of Patients with Anti-HMGCR Antibodies. The anti-HMGCR antibody positive patients were clinically described in a previous study [11]. Briefly, their mean age was 54.4 years (range from 16 to 84; standard deviation 21.1 years) and 16/20 (80.0%) were females. In 18/20 (90.0%) of the patients, a diagnosis of necrotizing myopathy was established. The two remaining patients were not diagnosed at the last clinical follow-up but are highly suspected to suffer from myositis. The mean age at disease onset was 48.5 (SD 20.1 years, range 12–83 years). Nine out of 20 (45%) anti-HMGCR positive patients were on statin. All patients with anti-HMGCR antibodies were negative for all autoantibodies tested (SS-A, SS-B, Sm, RNP, Jo-1, Scl-70, Centromere, Mi2, PM/Scl, Ku, PL-7, PL-12, and SRP).

3.3. Extended Specificity Study of the ELISA and Indirect Immunofluorescence Pattern on HEp-2 Cells. Testing various controls showed high specificity (99.3%). 3/518 apparently healthy individuals and 3/117 patients with Sicca Syndrome were positive (see Figure 3); they all had low titers of anti-HMGCR antibodies. To investigate the staining pattern of anti-HMGCR antibodies a strongly reactive patient sample was used to stain HEp-2 cells. On these cells, the fluorescence pattern suggestive for anti-HMGCR antibodies is a finely granular cytoplasmic staining on a minority (3% or less) of HEp-2000 cells with perinuclear reinforcement (see Figure 4).

3.4. Epitope Mapping. Several potential epitopes were identified using the solid phase peptide arrays (see Figure 5). A total of six sequences were selected based on surface exposure (^{531}GYMPIPVGVAGPL^{543}, ^{748}GYNAHAANIVTAI^{760}, ^{554}MATTEGCLVASTN^{566}, ^{689}TDKKPAAINWIEG^{701}, ^{561}CLVASTNRGCRAI^{573}, and

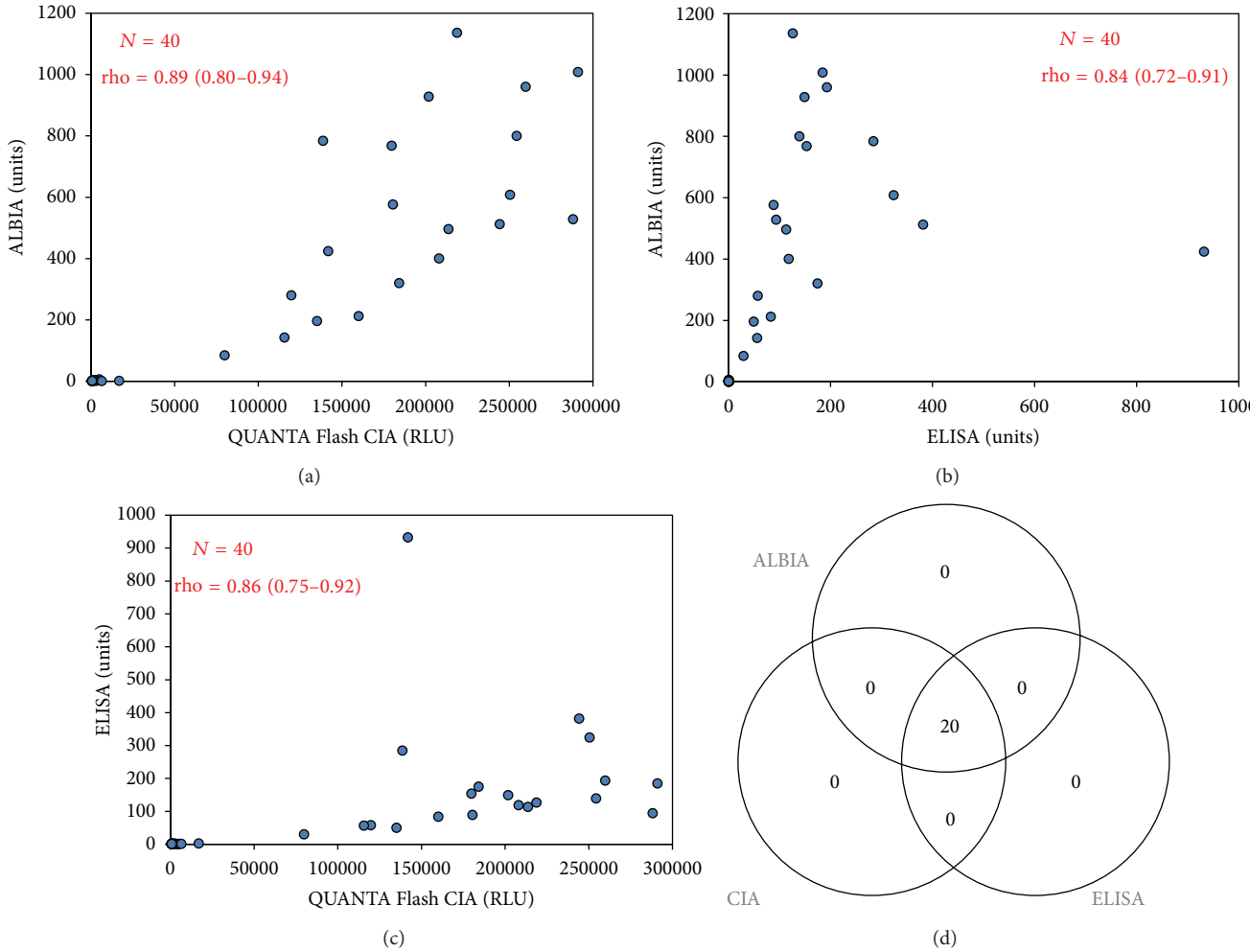


Figure 2: Correlation of anti-HMGCR antibodies detected using different methods. Spearman correlation diagrams are shown in (a)–(c) and a Venn Diagram in (d). All assays show good qualitative and quantitative agreements. ALBIA: addressable laser bead assay; CIA: chemiluminscent immunoassay; RLU: relative light units.

^{702}RGKSVVCEAVIPA^{714}), synthesized as soluble peptides and tested with positive sera and controls by ELISA. Reactivity found by solid phase peptide arrays could not be confirmed (data not shown).

4. Discussion

First described in 2010 [5], anti-HMGCR antibodies represent a promising biomarker to aid in the diagnosis and treatment decision of idiopathic inflammatory necrotizing myopathies (IINM) [1]. The present study is the first to compare different methods for the detection of anti-HMGCR antibodies (ALBIA, ELISA, and CIA), two of them manufactured in a precommercial setting and one based on ALBIA. All three assays demonstrated very good agreement. Both, the ELISA and the CIA, use a 63 kDa fragment of HMGCR which has previously been described as the epitope containing region of the antibodies [6]. In addition, we studied the coexistence of anti-HMGCR antibodies with other MSA and MAA and found that in our patients anti-HMGCR antibodies were the only detectable autoantibody. Our data on the association between statin use and anti-HMGCR antibodies confirms previous results [6]. We also confirm that the majority of patients with anti-HMGCR antibodies have IINM [5]. Anti-HMGCR antibodies might become available as a single assay on a fully automated analyzer [13], as ELISA and/or as part of multiparameter assays.

A recent study showed that the majority of patients with and without statin exposure, including those with self-limited statin intolerance, do not develop anti-HMGCR antibodies [17]. Therefore, anti-HMGCR antibodies are highly specific for those with an IIM. To further analyze the specificity especially against healthy and diseased controls, we performed a specificity study by ELISA. Using various disease controls (n = 824), very high specificity (99.3%) of the ELISA test was demonstrated. All six anti-HMGCR antibody positive control samples had low titers. Therefore, anti-HMGCR antibodies detected by ELISA, especially those moderate and high titers, are highly indicative of IINM.

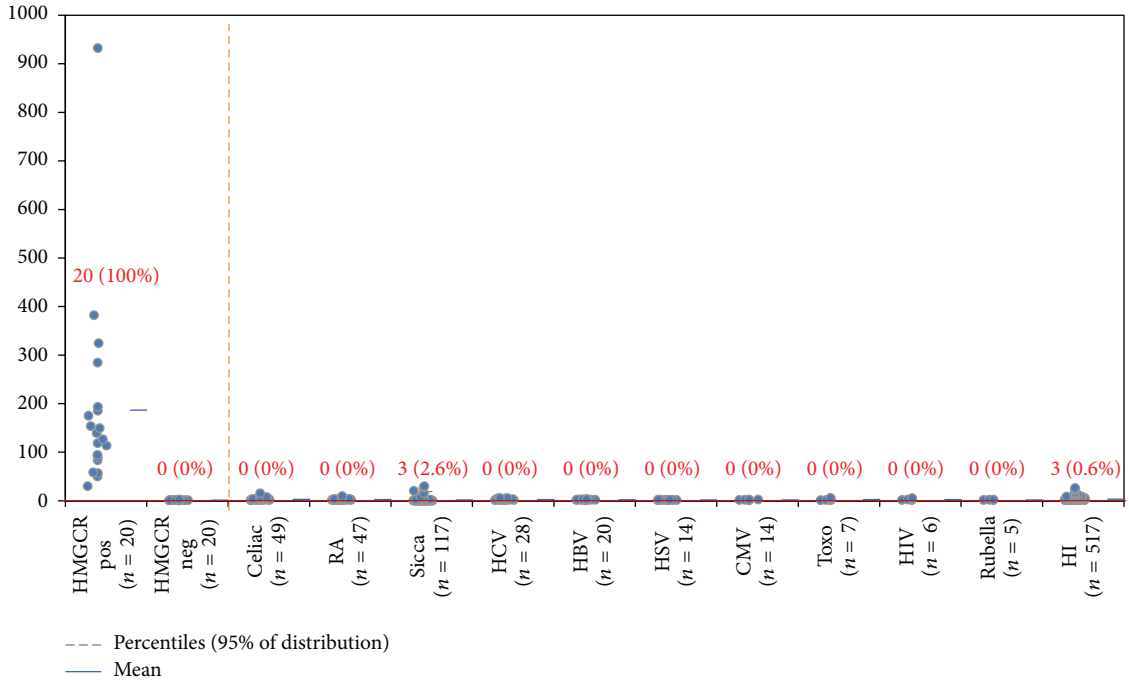


Figure 3: Comparative descriptive analysis of anti-HMGCR antibodies detected by ELISA. On the left side of the orange dotted line samples identified based on addressable laser bead assay are shown. On the right side of the dotted line, the results of various disease controls are displayed. Anti-HMGCR antibodies detected in various disease cohorts using ELISA show high disease specificity (99.3%).

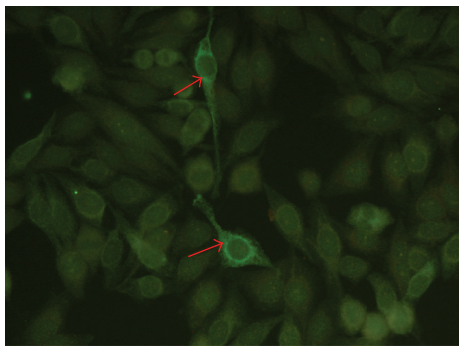


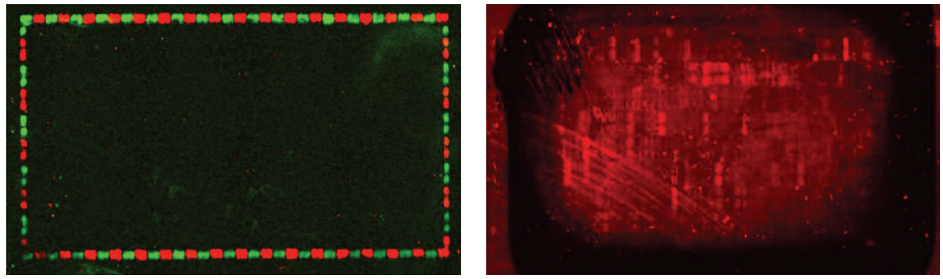
Figure 4: Putative indirect immunofluorescence pattern on HEp-2 cells. A serum sample from a patient with anti-HMGCR antibodies was used to stain HEp-2 cells. On these cells, the fluorescence pattern suggestive for anti-HMGCR is a finely granular cytoplasmic staining on a minority (3% or less) of HEp-2000 cells with perinuclear reinforcement.

Recently, an increasing prevalence of IINM was reported. In about half of the identified patients (45%) no autoantibody could be detected. The majority of those patients were statin users (67%) compared to 18% in the group with detectable autoantibodies (i.e., Jo-1, SRP, PM/Scl, and Ro52) [18]. Future studies are mandatory to investigate the pathophysiological mechanism of anti-HMGCR antibodies and the root cause for the increasing prevalence IINM.

Although we could not find any other autoantibody in our patients with anti-HMGCR reactivity, we cannot rule out that the patients have autoantibodies that have not been tested in our study (i.e., anti-TIF1 families, anti-MDA5, anti-NXP2, and so forth).

Autoantibodies to intracellular antigens (referred to as antinuclear antibodies) are commonly tested using IIF on HEp-2 cells to aid in the diagnosis of systemic autoimmune diseases including myositis [19]. Consequently, we wanted to study if anti-HMGCR antibodies decorate certain structure on HEp-2 cells. We found that patients with anti-HMGCR antibodies frequently stain cytoplasmic structures, however, not in all cells. In this context it is important to point out that the sensitivity for antibodies to cytoplasmic antigens (i.e., Jo-1 or ribosomal P) is limited [20]. In summary, further studies are needed to (1) confirm the observed staining pattern, to (2) analyze the pattern on slides from different manufacturers and to (3) assess the reliability (sensitivity) of IIF HEp-2 for the detection of anti-HMGCR antibodies.

Using solid phase peptide synthesis, we found several peptides reacting with anti-HMGCR antibodies contained in a serum of a patient with statin-associated necrotizing myopathies. In order to confirm the reactivity using a different method, we selected surface exposed epitope sequences for synthesis of soluble peptides. Although we were unable to confirm the peptide reactivity using soluble HMGCR derived peptides, it cannot be ruled out that anti-HMGCR antibodies bind linear epitopes. However, based on our data it is likely that conformational structures play a role in the formation of the major epitope. Further studies are needed to analyze the nature of the epitope on HMGCR and to address the reason for the discrepant results between the two methods



(a) (b)

High signal intensities	Medium signal intensities
TETESTLSLKNPI	SLKNPITSPVVTQ
STLSLKNPITSPV	FVVGNSSLLDTSS
ITSPVVTQKKVPD	SLLDTSSVLVTQE
NQKCDSVEEETGI	PLCLDEKEFQVPM
GYMPIPVGVAGPL	LETSEGFAVIKEA
EKEFQVPMATTEG	LSKLHEYFPEMQI
MATTEGCLVASTN	
CLVASTNRGCRAI	
SAEVKAWLETSEG	
EKALSKLHEYFPE	
TDKKPAAINWIEG	
RGKSVVCEAVIPA	
GYNAHAANIVTAI	

(c)

Figure 5: Epitope mapping of anti-HMGCR antibodies. DyLight680 showed no background interaction with the secondary antibody (a); incubation with anti-HMGCR positive serum revealed a number of reactive peptides (b). Epitopes identified using solid phase array are shown in (c). Sequences in yellow were selected for soluble peptide synthesis based on reactivity and surface exposure.

for the detection of antibodies to synthetic peptides. Different technologies including phage or bacterial display, synthetic peptides, or recombinant proteins might prove useful [19].

5. Conclusions

Anti-HMGCR antibodies can be detected using different methods with good intermethod agreement. Anti-HMGCR antibodies are strongly associated with IINM and to a lesser extent with statin use. Testing for anti-HMGCR antibodies might prove useful in the diagnosis of IIM and in the differentiation between self-limited and persistent statin associated myopathy which requires long-term immunosuppressive treatment.

Abbreviations

ALBIA: Addressable laser bead immunoassay
HMGCR: 3-Hydroxy-3-methylglutaryl-coenzyme A reductase
IIM: Idiopathic inflammatory myopathies
IINM: Idiopathic inflammatory necrotizing myopathies
MAA: Myositis associated antibodies
MSA: Myositis specific antibodies
SARD: Systemic autoimmune rheumatic disease
SRP: Signal recognition particle.

Conflict of Interests

M. Mahler, J. Phillips, and R. Fowler are employed at INOVA diagnostics selling autoantibody assays. A. Mammen is an inventor of the patent and receives royalties. The other authors have no conflict of interests.

Acknowledgments

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Clinical Study

High Prevalence of Antinuclear Antibodies in Children with Thyroid Autoimmunity

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Background. Antinuclear antibodies (ANA) are a hallmark of many autoimmune diseases and can be detected many years before disease onset. Autoimmune thyroid diseases (AITD) are frequently associated with other organ- and non-organ-specific autoimmune disorders. *Objectives*. To assess the prevalence of ANA in pediatric patients with AITD and their clinical correlations. *Methods*. Ninety-three consecutive pediatric patients with AITD were enrolled (86 children with chronic lymphocytic thyroiditis and 7 with Graves' disease). ANA, anti-double DNA (anti-dsDNA) antibodies, anti-extractable nuclear antigen (anti-ENA), anti-cyclic citrullinated peptide antibodies (anti-CCP), and rheumatoid factor (RF) was obtained. Signs and symptoms potentially related to rheumatic diseases in children were investigated by a questionnaire. *Results*. ANA positivity was found in 66/93 children (71%), anti-ENA in 4/93 (4.3%), anti-dsDNA in 1/93 (1.1%), RF in 3/93 (3.2%), and anti-CCP in none. No significant differences were found between the ANA-positive and ANA-negative groups with respect to age, sex, L-thyroxine treatment, or prevalence of other autoimmune diseases. Overall, parental autoimmunity was found in 23%. *Conclusions*. ANA positivity was demonstrated in 71% of children with AITD. ANA positivity was not related to overt immune-rheumatic diseases. However, because the positivity of ANA can occur even many years before the onset of systemic autoimmune diseases, prospective studies are warranted.

1. Introduction

Antinuclear antibodies (ANA) are a marker of several autoimmune diseases including autoimmune thyroid disease (AITD) and Systemic Lupus Erythematosus (SLE) and they can be detected many years before disease onset [1–5]. ANA positivity can also be found in patients with malignant or infectious diseases as well as in healthy subjects [2–5].

The AITD, Graves' disease (GD), and chronic lymphocytic thyroiditis (CLT) are organ-specific autoimmune disorders that are defined by lymphocytic infiltration of the thyroid [6] and autoantibodies against thyroid antigens [i.e., thyroid peroxidase antibody (TPOAb), thyroglobulin antibody (TgAb), and anti-TSH-receptor antibody (TRAb)] [7]. AITD is frequently associated with other organ and non-organ-specific autoimmune disorders [8–10]. This association has been also reported in juvenile forms of chronic arthritis and SLE [11, 12]. ANA prevalence up to 45% has been reported in adult patients with AITD [13, 14]. To the best of our knowledge, so far, only one study analyzed ANA positivity in children with AITD [15]. We investigated the prevalence of serum ANA in pediatric patients with AITD and their association with signs and symptoms related to immune-rheumatic diseases.

2. Patients and Methods

We studied 93 consecutive children (86 patients with CLT and 7 with GD, 76 females) referred for AITD to the Pediatric Endocrinology Unit of "Sapienza" University in Rome. CLT was defined on the basis of the presence of thyroid autoantibodies more than two times the upper normal value (nv) ([TPOAb nv < 20 IU/mL] and/or TgAb

[nv < 50 IU/mL]) and thyroid ultrasound evaluation showing reduced echogenicity compatible with thyroiditis, regardless of thyroid function. GD was defined by clinical and biochemical hyperthyroidism and by positivity for TRAb.

Written informed consent was obtained from parents. This study was approved by the ethical committee of our institution.

The enrolled patients underwent a complete physical examination and the clinical and laboratory data were collected in a standardized form, which includes demographics, past medical history with date of diagnosis, comorbidities, and previous and concomitant treatments. All children and parents were interviewed according to a questionnaire seeking signs and symptoms related to rheumatic diseases in children. The questionnaire took into account the following signs and symptoms: joint pain, joint swelling, back pain, morning stiffness, asthenia, Raynaud's phenomenon, xerostomia, xerophthalmia, pleuritis, and pericarditis. Children with signs and/or symptoms suggestive of immune-rheumatic diseases were examined by a rheumatologist (FC).

Patients underwent peripheral blood sample collection and sera were stored at −20°C until being assayed.

Free T3, free T4, TSH, TPOAb, and TgAb serum levels were determined in all patients and TRAb were determined in presence of hyperthyroidism. FT3 and FT4 were determined by RIA and TSH (upper normal value 3.5 μU/mL) was determined by immunoradiometric assay (all by Byk Sangtec Diagnostica, Dietzbach, Germany). TRAb were detected by radioreceptor assay (Radim, Angleur, Belgium) and TPOAb and TgAb by immunoradiometric assay (ICN Pharmaceutical Inc., Costa Mesa, CA).

ANA were detected by indirect immunofluorescence assay (IIFA) on HEp-2 cells (ANA Nova LIte TM HEp-2, INOVA Diagnostics Inc., San Diego, CA 92131, USA). Anti-double DNA antibodies (anti-dsDNA) were investigated by *Crithidia luciliae* immunofluorescence test (CLIFT) (A. Menarini Diagnostics, 50131 Florence, Italy). ANA were considered positive at a titer ≥ 1 : 80, anti-dsDNA at a titer ≥ 1 : 10. Immunofluorscence intensity ranged from + to ++++. Serum antibodies against extractable nuclear antigen (anti-ENA screening and in the case of ENA positivity: Sm, RNP, SS-A, SS-B, Scl-70, Jo-1) were determined by ELISA (QUANTA Lite ENA 6, INOVA Diagnostics Inc., San Diego, CA 92131, USA). In addition, anti-cyclic citrullinated peptide antibodies (anti-CCP) and rheumatoid factor (RF) were determined. Anti-CCP were determined by fluoroenzyme-immunoassay (EliACCP), using immunoCAP 100 analyzer (Phadia AB, 75002 Uppsala, Sweden) and RF was determined by nephelometry using BN2 automate (N Latex RF kit, Siemens Healthcare Diagnostics Products, Marburg 35037, Germany).

3. Statistics

Statistical calculations were performed using SPSS for Windows Version 17 (SPSS, Chicago, IL, USA). Data were expressed as mean and standard deviation for continuous data. Chi-square or Fisher's exact test was carried out when appropriate. P values < 0.05 were considered statistically significant.

4. Results

Clinical and laboratory features of the enrolled patients are reported in Table 1(a). ANA positivity was found in 66/93 children (71%), anti-ENA was found in 4/93 (4.3%), anti-dsDNA antibodies were found in 1/93 (1.1%), RF was detectable in 3/93 (3.2%), and anti-CCP were found in none. ENA-specific autoantibodies were determined in 3 out of 4 anti-ENA-positive patients, 1 had anti-RNP positive, 1 had anti-Jo-1, and 1 was negative for specific autoantibodies. The ANA pattern was homogeneous in 61/66 (92.4%), coarse/fine speckled in 4/66 (6%), and nucleolar in 2/66 (3%). The IIFA intensity was +++ in 23/66 (34.8%), ++ in 24/66 (36.3%), and + in 19/66 (28.7%) cases.

Among the 93 children with AITD, 20 (21%) had at least one other autoimmune disease: 9 with celiac disease (CD), 2 with autoimmune gastritis (AG), 4 with type 1 diabetes mellitus (type 1 DM), 2 with alopecia, 2 with vitiligo, and 1 with autoimmune thrombocytopenia. We found that 3/7 (43%) of children with GD presented with one other autoimmune disease (1 CD, 1 type DM, and 1 AG), versus 17/86 (20%) with CLT (8 CD, 1 AG, 3 type 1 DM, 2 alopecia, 2 vitiligo, and 1 autoimmune thrombocytopenia). The difference in the frequency of autoimmune disease associated with CLT versus GD was not significant.

Clinical and laboratory features of ANA-positive and ANA-negative patients are compared in Table 1(b). No significant differences were found between the ANA-positive and ANA-negative groups with respect to age, sex, L-thyroxine treatment, TSH, TPOAb, and TgAb levels, or presence of other autoimmune diseases in them and in their parents. Associated autoimmune diseases in ANA-positive and ANA-negative children and their parents are reported in Table 2. Hereby ANA-positive children did not differ for the frequency of additional autoimmune disease, but there was a higher percentage of parental autoimmunity in ANA-negative patients, however, without significance.

Signs and symptoms as investigated by questionnaire are detailed in Table 3. No significant differences were found between ANA-positive and ANA-negative children with AITD.

Children with persistent joint pain were referred to a rheumatologist (FC). Children with Raynaud's phenomenon underwent capillaroscopy. No evidence of SLE, RA, or other systemic autoimmune diseases was found.

5. Discussion

AITD is a common autoimmune disease and it is frequently associated with other organ and non-organ-specific autoimmune disorders [8–10]. A variable ANA prevalence up to 45% has been reported in AITD adult patients [13, 14].

ANA can also be detected in different autoimmune disorders (i.e., SLE, Sjogren's syndrome, progressive systemic sclerosis, mixed connective-tissue disease, juvenile idiopathic

Table 1: (a) Clinical and laboratory features of the patients at inclusion. (b) Clinical and laboratory features of ANA-positive and ANA-negative children.

(a)

Characteristic	Patients ($N = 93$)
F/M	76/17
CLT (N)	86
GD (N)	7
Age at diagnosis (mean years ± SD)	10.2 ± 3.9
Age at sampling (mean years ± SD)	12.1 ± 4.86
Duration of disease (mean years ± SD)	2.79 ± 3.67
CLT on LT4 treatment (N/%)	47/86 (54.6%)
TSH (μIU/mL ± SD)	2.8 ± 1.8
TPOAb (IU/mL ± SD)	819 ± 908
TgAb (IU/mL ± SD)	512 ± 605
Associated autoimmune disease in children (N/%)	20/93 (21%)
Associated autoimmune disease in CLT (N/%)	17/86 (20%)
Associated autoimmune disease in GD (N/%)	3/7 (43%)
Autoimmune disease in parents (N/%)	43/186 (23%)

(b)

Patients	ANA positive ($N = 66$)	ANA negative ($N = 27$)	P
F/M	56/10	20/7	
CLT (N/%)	62 (72%)	24 (28%)	0.7
GD (N%)	4 (57%)	3 (43%)	0.7
Age at diagnosis (mean years ± SD)	10.4 ± 3.8	9.9 ± 4.2	0.61
Age at sampling (mean years ± SD)	12.4 ± 4.8	11.4 ± 4.4	0.32
TSH (μIU/mL ± SD)	3.01 ± 1.77	2.48 ± 1.86	0.2
TPOAb (IU/mL ± SD)	903 ± 941	614 ± 803	0.61
TgAb (IU/mL ± SD)	519 ± 593	494 ± 666	0.85
LT4 therapy (CLT) (N/%)	31/62 (50%)	16/27 (66%)	0.99

F/M: females/males; CLT: chronic lymphocytic thyroiditis; GD: graves' disease; TSH: thyroid stimulating hormone; TPOAb: thyroid peroxidase antibodies; TgAb: thyroglobulin antibodies; LT4: L-thyroxine.

arthritis, primary autoimmune cholangitis, and autoimmune hepatitis) as well as in infections (2,4). In particular, ANA can be detected in over ninety percent of patients with SLE, a multifactorial autoimmune disease, involving genetic and environmental factors, characterized by a wide range of autoantibodies and clinical manifestations [4, 16–25].

ANA can be also found in healthy people [2]. A recent cross-sectional analysis of 4754 individuals older than 12 years showed a prevalence of ANA of 13.8% [26]. A similar prevalence of 12.6% was reported in healthy children, with higher titers found between 5 and 10 years of age [27]. To our knowledge, only one previous study investigated ANA prevalence in children with AITD. The authors, using a cut-off of 1 : 40 for ANA IIFA on HEp-2 cells, demonstrated an incidence of ANA positivity significantly higher in patients with untreated GD (71%) than in CLT (33%) [15]. In addition, ANA positivity was identified as a predictive factor for poor response to antithyroid drugs in GD. In our study, CLT represented 92% of total enrolled patients versus 36% in the previous study. Since we analyzed GD regardless of treatment and we included only 7 children affected with GD, we were not able to correlate GD activity with ANA positivity. In CLT patients, we did not find any difference in L-thyroxine treatment between ANA-positive and ANA-negative children. In contrast, a correlation between increased ANA levels and reduced thyroid volumes was reported in adult patients affected by vitiligo and AITD [28].

To detect serum ANA, we used the same method of Inamo and Harada [15], IIFA on HEp-2 cells, that is considered the most reliable method to search ANA [2, 29]. Using a higher cut-off value of 1 : 80, we found detectable ANA in 71% of children with AITD and the IIFA homogeneous pattern was detected in 92% of cases. Titers and pattern of ANA have been proposed to be a critical parameter for discriminating ANA, a homogeneous pattern being suggestive of rheumatic diseases [30]. A nuclear fine speckled pattern was reported in ANA-positive healthy children in 77% of cases [27].

We did not find any significant difference in ANA-positive and ANA-negative group with respect to age, sex, LT4 treatment, TSH, TPOAb and TgAb levels, and prevalence of other autoimmune diseases in the children or in the parents. No differences were detected investigating

Table 2: Associated autoimmune diseases in ANA-positive and ANA-negative children with AITD and in their parents.

Patients	ANA positive ($N = 66$)	ANA negative ($N = 27$)	P
Associated autoimmune diseases (N/%)	14/66 (21%)	6/27 (22%)	0.87
Celiac disease (N)	6	3	
Type 1 DM (N)	2	2	
Autoimmune gastritis (N)	2	0	
Vitiligo (N)	2	0	
Alopecia (N)	1	1	
Autoimmune thrombocytopenia (N)	1	0	
Parents	66 mothers, 66 fathers	27 mothers, 27 fathers	
Autoimmune diseases in parents (N/%)	27/132 (20.4%)	16/54 (29.6%)	0.24
Autoimmune diseases in mothers (N)	18	10	
AITD (N)	14	10	
SLE (N)	1	0	
Sjogren's syndrome (N)	1	0	
Autoimmune thrombocytopenia (N)	1	0	
Autoimmune diseases in fathers (N)	9	6	
AITD (N)	8	4	
Vitiligo (N)	1	1	
Rheumatoid arthritis (N)	0	1	

Type 1 DM: type 1 diabetes mellitus; AITD: autoimmune thyroid disease; SLE: systemic lupus erythematosus.

Table 3: Positive answers at questionnaire in ANA-positive and ANA-negative children with AITD.

Question	ANA positive ($N = 66$)	ANA negative ($N = 27$)	P
Joint pain	12 (18%)	5 (18%)	0.87
Joint swelling	0 (0%)	0 (0%)	—
Morning stiffness	1 (1.5%)	0 (0%)	—
Back pain	1 (1.5%)	1 (3.7%)	—
Asthenia	14 (21%)	4 (14%)	0.47
Raynaud's	3 (4.5%)	1 (3.7%)	0.63
Xerostomia	3 (4.5%)	1 (3.7%)	0.63
Xerophthalmia	3 (4.5%)	1 (3.7%)	0.63
Pleuritis	0	0	—
Pericarditis	0	0	—

signs and symptoms of systemic diseases between ANA-positive and ANA-negative group. Altogether, joint pain was referred by 19% of patients, asthenia was referred by 19%, and Raynaud's phenomenon was present in 4.3%, as well as xerostomia and xerophtalmia. Because of the fact that hypothyroidism itself can cause symptoms as asthenia, we confirmed symptoms when children were euthyroid. Torok and Arkachaisri [31] in children and adolescents referred to a rheumatological center for ANA positivity and investigated for AITD, found arthralgias and fatigue more frequently in ANA-positive/antithyroid antibodies-negative subjects. The prevalence of positive antithyroid antibodies in this study was 30% versus 3-4% in general pediatric population [32], and hypothyroidism was found in 14%. It may suggest that to screen for AITD may be worthwhile in apparently healthy children with ANA positivity.

The clinical and biological meaning of ANA is still debated [29, 33, 34]. It has been speculated that a defect in the mechanisms involved in the engulfment of dead cell with inappropriate clearance of self-nucleic acids can cause autoimmune diseases. A deficiency of clearance of apoptotic cells is considered one of the causes of SLE and may underlie autoimmunity itself [35–37]. Activation of the innate and acquired immune response, which can be induced by infection, inflammation, or tissue injury, may impact on the development of autoimmunity in the thyroid [38, 39]. We can speculate that such a high prevalence of ANA in juvenile AITD may be read as a link to the involvement of self nucleic acids in the development of AITD.

An important point of our study is the observation of parental autoimmunity in up to 30% with only slightly lower percentage (20%) in ANA-positive children. Whether this reflects (epi-)genetic heritability or a shared familial environment triggering autoimmunity in parents and children needs to be investigated in prospective family studies.

In conclusion, although we analyzed a limited number of cases, our study demonstrated ANA positivity in over 70% of children with AITD. ANA positivity at the time of the study was not related to overt immune-rheumatic diseases.

In our opinion, the finding of ANA positivity in children and adolescents affected by AITD needs a careful reevaluation: it can be interpreted as a manifestation of "activated autoimmunity" without clinical relevance at the time of the study. However, because the positivity of ANA and other non-organ-specific autoantibodies can occur even many years

before the onset of systemic autoimmune diseases [5, 40, 41], we think that prospective studies are warranted, especially in subjects positive for anti-dsDNA and anti-ENA.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

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Research Article

Interpretation of ANA Indirect Immunofluorescence Test Outside the Darkroom Using NOVA View Compared to Manual Microscopy

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Objective. To evaluate NOVA View with focus on reading archived images versus microscope based manual interpretation of ANA HEp-2 slides by an experienced, certified medical technologist. *Methods.* 369 well defined sera from: 44 rheumatoid arthritis, 50 systemic lupus erythematosus, 35 scleroderma, 19 Sjögren's syndrome, and 10 polymyositis patients as well as 99 healthy controls were examined. In addition, 12 defined sera from the Centers for Disease Control and 100 random patient sera sent to ARUP Laboratories for ANA HEp-2 IIF testing were included. Samples were read using the archived images on NOVA View and compared to results obtained from manual reading. *Results.* At a 1 : 40/1 : 80 dilution the resulting comparison demonstrated 94.8%/92.9% positive, 97.4%/97.4% negative, and 96.5%/96.2% total agreements between manual IIF and NOVA View archived images. Agreement of identifiable patterns between methods was 97%, with PCNA and mixed patterns undetermined. *Conclusion.* Excellent agreements were obtained between reading archived images on NOVA View and manually on a fluorescent microscope. In addition, workflow benefits were observed which need to be analyzed in future studies.

1. Introduction

The antinuclear antibody (ANA) test is a standard screening assay for detecting multiple antibodies that may be produced by a patient with an autoimmune or ANA associated rheumatic disease (AARD). Although there are several methodologies available to screen ANA, the American College of Rheumatology (ACR) issued a statement declaring HEp-2 indirect immunofluorescence (IIF) as the preferred method for ANA screening [1]. This declaration was based on the findings of a task force which investigated and collected information from physicians to evaluate nonstandardization of the various methodologies on the market for evaluating ANA. Using HEp-2 as a substrate allows the detection of more than 100 autoantibodies to different nuclear and cytoplasmic antigens [2]. These include antibodies to dsDNA, SS-A, Ro52, SS-B, RNP, centromere, Scl-70, Jo-1, ribosomal P, RNA Polymerase III, PM/Scl, Ku, Th/To, and Mi-2 to term the most important antigens. There are 5 to 6 indirect immunofluorescence (IIF) nuclear patterns that are commonly reported by most laboratories, namely, homogeneous, speckled, nucleolar, centromere, peripheral/rimmed, and proliferating cell nuclear antigen (PCNA). Laboratories performing the ANA IIF test typically report a positive result with a pattern and titer. This aids the physician when deciding what tests to order next, if any.

Performing IIF test is labor intensive, subjective, and prone to reader bias [3–7]. Many other variables affect the IIF test result such as the HEp-2 substrate, conjugate, microscope, type of bulb, and bulb life [2, 8–14]. Over the

past few decades as newer technologies emerged for testing ANAs, there were fewer and fewer laboratorians with the knowledge and skill to perform ANA IIF testing. As a reference laboratory, ARUP continues to offer and perform HEp-2 IIF for ANA testing. Extensive time is required to train a technologist to be competent in reading and interpreting ANA IIF testing. In addition, there is a need for standardization and automation in ANA testing [1–3, 15].

Autoimmune laboratories have made strides in automation over the past decade but are still far behind other areas of the laboratory, such as chemistry with their fully automated instrumentation. Automated pattern interpretation of HEp-2 ANA was first described in 2002 by Perner et al. [16] Since then, there have been several studies of automated or digital IIF interpretation for positive and negative discrimination. Some systems incorporate pattern recognition algorithms. All conclude that automated IIF analysis will improve inter- and intralaboratory results [17–25]. The NOVA View instrument (INOVA Diagnostics, Inc., San Diego, CA) has been designed to address this need. NOVA View is an automated digital image analysis system, which is used for acquiring, analyzing, and interpreting ANA testing on HEp-2 cells, based on measured Light Intensity Units (LIU) and pattern recognition. NOVA View results are expressed in LIU and interpreted as negative or positive based on a preset cutoff. The cutoff intensity is preset by INOVA and may be adjusted for the customer based on their patient population and performance goals. The patented process produces three to five images per patient sample. The automated scan is followed by visual verification of the digital images, allowing for either confirmation or revision of results by the operator. NOVA View software recognizes five basic patterns: homogeneous, speckled, centromere, nucleolar, and nuclear dots. Pattern recognition is based on a software algorithm that analyzes the intensity and distribution of the fluorescent light over the area of the nuclei based on specific criteria. Mixed patterns may not be recognized by the software and may be reported as "unrecognized." In these cases the final pattern is determined by the user during the revision and confirmation of the digital images.

Based on the recommendation of the ACR for the use of HEp-2 IIF to test for ANA, we aimed to compare the agreement of the NOVA View archived images to the interpretation of the same samples on a manual fluorescent microscope interpreted by a certified medical technologist, with emphasis on agreement of end point titer. In addition, the data were used to calculate ANA titers and positivity rate in various AARD.

2. Materials and Methods

2.1. Clinical Samples. Clinically defined serum samples from patients suffering from SLE ($n = 50$), rheumatoid arthritis (RA, $n = 44$), SSc ($n = 35$), Sjögren's syndrome (SjS, $n = 19$), and polymyositis (PM, $n = 10$) were included. Diagnoses were established as previously described or according to the respective disease classification criteria [26]. In addition, 99 healthy adult donor sera which consisted of 70% female and 30% male between the ages of 19 to 59 years of age were tested.

2.2. CDC ANA Reference Panel. International reference serum panel (CDC ANA #1–12) was obtained from the Centers of Disease Control and Prevention (CDC) (http://asc.dental.ufl.edu/ReferenceSera.html). (Biological Reference Reagents, NCID/SRP/BRR, Mailstop C-21, Centers for Disease Control and Prevention (CDC), 1600 Clifton Rd. N.E., Atlanta, GA, U.S.A).

2.3. Consecutive Routine Samples. Lastly, 100 consecutive samples from an individual client, sent to ARUP Laboratories for ANA IIF testing, were reviewed. All patient samples included in the study were deidentified according to the University of Utah Institutional Review Board-approved protocol number 7275 to meet the Health Information Portability and Accountability Act Patient Confidentiality Guidelines.

2.4. Microscopes and Indirect Immunofluorescence Reagents. NOVA Lite HEp-2 IgG ANA with DAPI kit and the NOVA View instrument with 1.0.2 software containing a cut-off value of 100 LIU for positive results (INOVA Diagnostics, San Diego, CA). The conjugate used in this assay contains the usual FITC fluorophor along with diamidino-2-phenylindole (DAPI), a blue nuclear stain that selectively binds to double stranded DNA. DAPI allows the instrument to "find" the cells at a 400 nm wavelength. If the cell density is insufficient or there are no cells in the well, the instrument will not switch to FITC but will produce an "*X*," indicating an inadequate number or that no cells were found. Once the correct number of cells has been identified, the instrument switches to a 490 nm wavelength for FITC identification and quantification of antibody in the sample. NOVA View has 5 preselected fields where it collects a digital image producing 5 images on the screen when the sample is positive. These five preselected fields mimic the areas where a technologist would read with a manual microscope. If the sample is negative, three images are produced.

For the manual reading, a Nikon Eclipse 400 with an LED light source (ARUP Laboratories, Salt Lake City, Utah) was used.

All samples were processed manually and read on both the Nikon microscope and NOVA View, archived images with software version 1.0.2 by a board certified medical technologist. The technologist was blinded to sample classification and has 5 years of reading IIF daily at ARUP laboratories. Intensive training and continuous reading are needed for a technologist to accurately interpret HEp-2 ANA. At ARUP Laboratories, and other facilities, people who interpret HEp-2 ANA on clinical sera must be board certified. In order to read ANA IIF accurately and consistently they read daily and are challenged by internal and external surveys. Patterns recorded at ARUP include speckled, homogeneous, centromere, nucleolar, and nuclear dots, PCNA, and NuMA along with comments on cytoplasmic fluorescence observed.

3. Results

3.1. Agreement between Manual and NOVA View Interpretation. At a 1 : 40/1 : 80 dilution the resulting comparison

Table 1: NOVA View agreement with manual IIF: 1 : 40 screening dilution.

All patients (n = 369)	Manual IIF			Percent agreement (95% confidence)
	Positive	Negative	Total	
NOVA View				
Positive	128	6	134	Positive agreement = 94.8% (89.6–97.9%)
Negative	7	228	235	Negative agreement = 97.4% (94.5–99.1%)
Total	135	234	369	Total agreement = 96.5% (94.1–98.1%)
				κ = 0.92 (95% CI 0.88–0.96)

N: number of patients tested at a 1 : 40 dilution for HEp-2 ANA antibodies.
Manual results were read on a Nikon Eclipse 400 fluorescent microscope with an LED light source.
NOVA View confirmed results = archived images reviewed and confirmed by the operator.

Table 2: NOVA View agreement with manual IIF: 1 : 80 screening dilution.

All patients (n = 369)	Manual IIF			Percent agreement (95% confidence)
	Positive	Negative	Total	
NOVA View				
Positive	92	7	99	Positive agreement = 92.9% (86.0–97.1%)
Negative	7	263	270	Negative agreement = 97.4% (94.7–99.0%)
Total	99	270	369	Total agreement = 96.2% (93.7–97.9%)
				κ = 0.90 (95% CI 0.85–0.95)

N: number of patients tested at a 1 : 80 dilution for HEp-2 ANA antibodies.
Manual results were read on a Nikon Eclipse 400 fluorescent microscope with an LED light source.
NOVA View confirmed results = images reviewed and confirmed by the operator.

Table 3: Clinical sensitivity and specificity.

Assay	Sensitivity % (95% CI)	Specificity % (95% CI)	Excluding RA samples specificity % (95% CI)
Manual IIF, 1 : 40 dilution	60.5 (50.9–69.6)	89.5 (83.3–94.0)	93.9 (87.3–97.7)
NOVA View, 1 : 40 dilution	61.4 (51.8–70.4)	90.9 (85.0–95.1)	94.9 (88.6–98.3)
Manual IIF, 1 : 80 dilution	49.1 (39.6–58.7)	94.4 (89.3–97.6)	98.0 (92.9–99.8)
NOVA View, 1 : 80 dilution	50.9 (41.3–60.4)	94.4 (89.3–97.6)	97.0 (91.4–99.4)

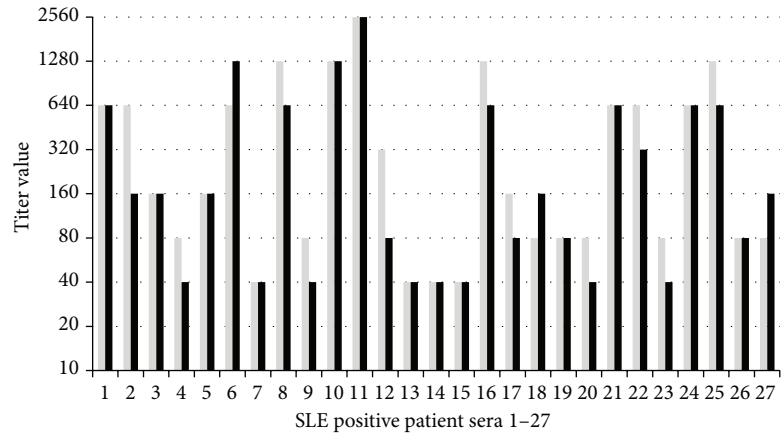
demonstrated 94.8%/92.9% positive, 97.4%/97.4% negative, and 96.5%/96.2% total agreements (Tables 1 and 2). The majority of discrepant results between the manual and the archived based interpretation were ±1 dilution difference. The highest fluctuation between results was seen at the 1 : 40 dilution. A total of 13 samples that were called positive by one method of reading and negative by the other all had titers of 1 : 40 or 1 : 80 and <1 : 40 (Table 1). One sample demonstrated a PCNA pattern, a pattern which is not recognized by the NOVA View system. Therefore we aimed to evaluate if this pattern can be identified as PCNA pattern by the technologist reading the NOVA View archived images. The archived image of the PCNA pattern was clear and easy to interpret.

Of the 100 samples sent to ARUP Laboratories for routine ANA testing, 63% were negative and 37% were positive. Titers were within plus or minus a doubling dilution between the manual and the NOVA View archived image results, and ranged from 1 : 40 to 1 : 2560 (Figure 1). Patterns matched 100%.

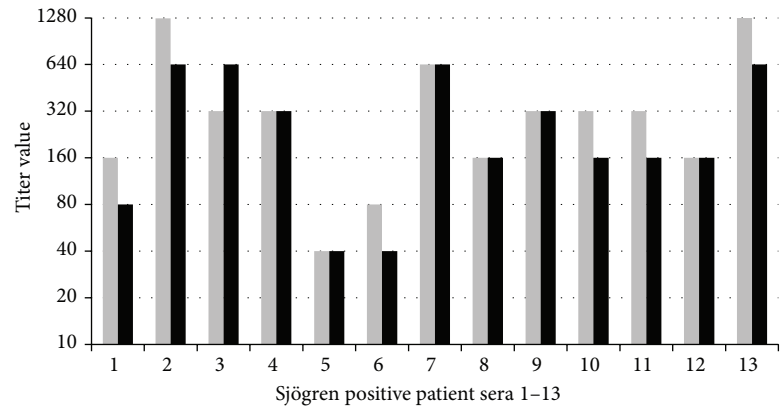
Good agreement and correlation between manual and NOVA View archived based reading were found. Results of the manual IIF interpretation were grouped into positive and negative. Subsequently, the titers obtained from the NOVA View archived image based interpretation were used to generate a receiver operating characteristic (ROC) curve showing very good agreement (Figure 2(a)). Spearman's correlation (all samples, n = 369) between IIF interpretation and NOVA View showed excellent correlation of rho = 0.96 (Figure 2(b)).

3.2. CDC Samples. The 12 CDC samples produced excellent correlation for pattern and titer (data not shown). All samples with ANA were positive with titers ranging from 1 : 40 to 1 : 320. The patterns match their original description of the antibody specificity.

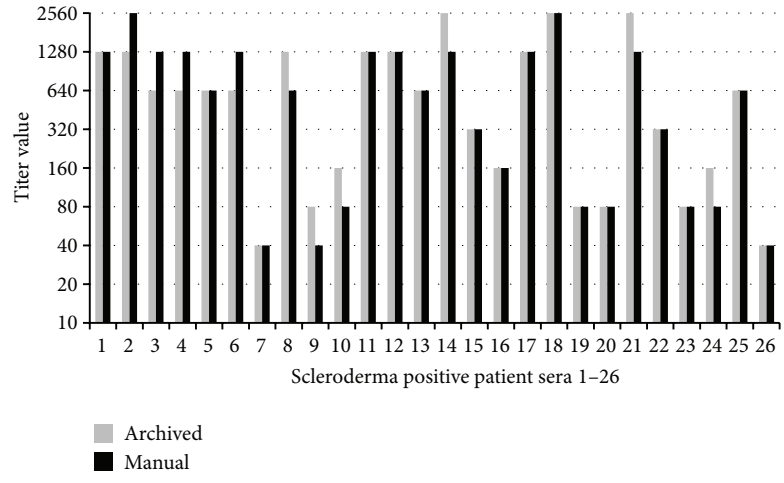
3.3. Clinically Defined Samples. The ROC analyses revealed similar discrimination between AARD and controls using the manual and the NOVA View archive reading (Table 3, Figure 3). A comparative descriptive analysis (Figure 4) demonstrated positivity in 56.0% of SLE, in 68.4% of SjS, in 74.3% of SSc, and in 30.0% of PM patients. In the control groups, 18.2% of the RA and 5.1% of the healthy



(a)



(b)



(c)

Figure 1: Antinuclear antibodies in different patient cohorts. (a) 27 positive systemic lupus erythematosus (SLE) patient sera titered at 1 : 40 through 1 : 2560. (b) Thirteen positive Sjögren's syndrome (SjS) patient sera titered at 1 : 40 through 1 : 1280. (c) 26 positive scleroderma patient sera titered at 1 : 40 through 1 : 2560. The gray bar represents the end point titer read on the NOVA View archived image. The black bar represents the titer read on a traditional manual microscope. All titers were read by the same technologist.

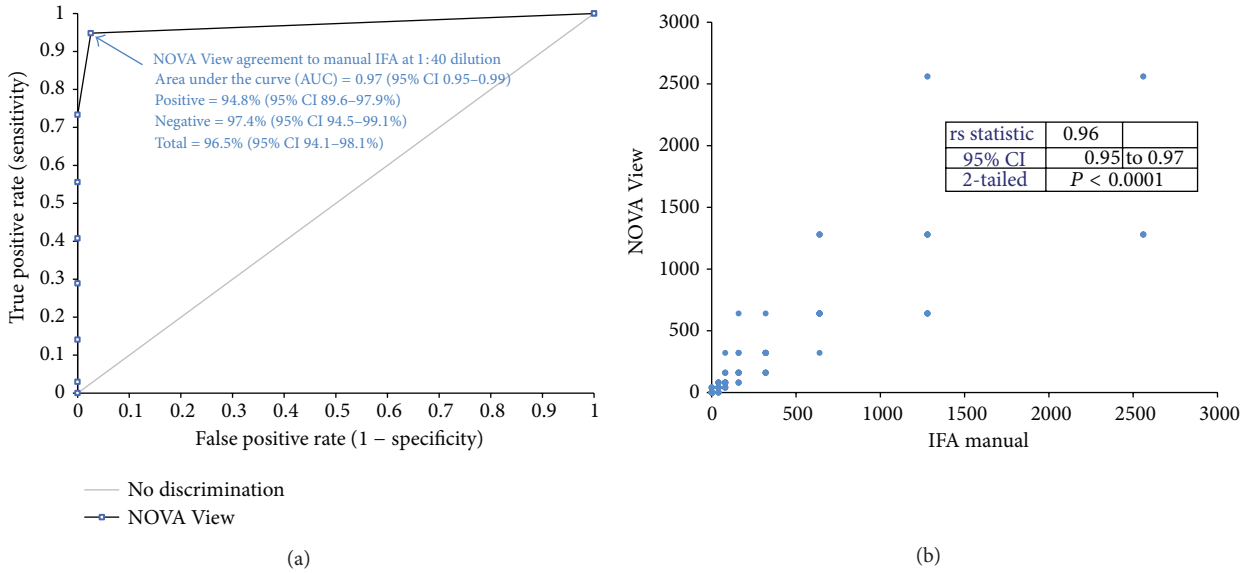


Figure 2: Agreement and correlation between manual and NOVA View archived image based reading. (a) Receiver operating characteristic (ROC) analysis. Results of the manual indirect immunofluorescence interpretation were grouped into positive and negative. Subsequently, the titers obtained from the NOVA View interpretation were used to generate a ROC curve showing very good agreement ($n = 369$). (b) Spearman's correlation (all samples, $n = 369$) between manual indirect immunofluorescence interpretation and NOVA View. Excellent correlation of rho = 0.96 was found.

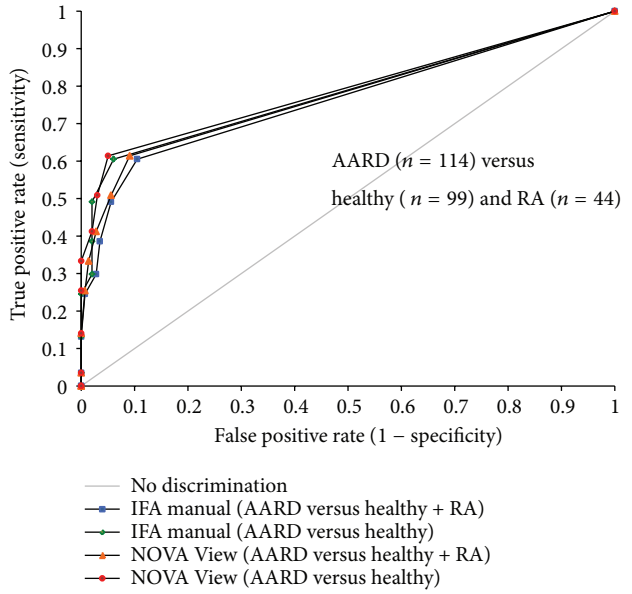


Figure 3: Comparative receiver operating characteristic (ROC) analyses. ROC for manual indirect immunofluorescence (IIF) manual interpretation and NOVA View results for ANA related autoimmune rheumatic disease (AARD) versus healthy controls and rheumatoid arthritis (RA). The ROC curves were similar for manual IIF and NOVA View, and as expected, specificity improves for both methods when RA patients are removed from analysis.

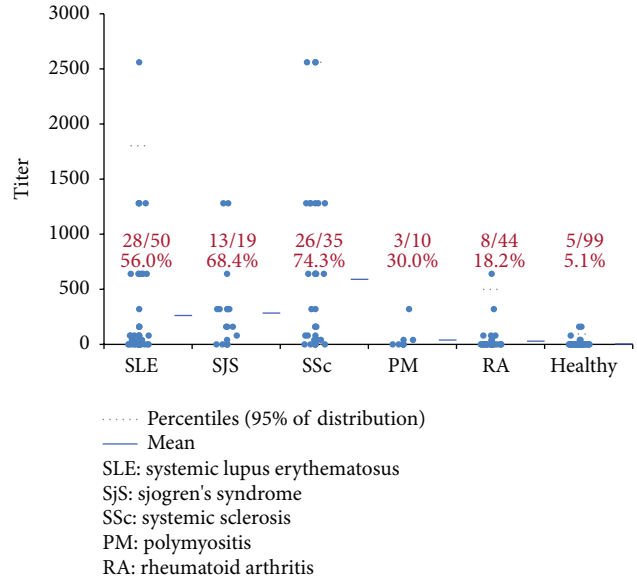


Figure 4: Antinuclear antibody titer and prevalence among ANA associated rheumatic diseases (AARD, $n = 114$), rheumatoid arthritis (RA, $n = 44$), and healthy controls ($n = 99$). Results are based on NOVA View archived images (similar results were found using manual reading).

individuals were ANA positive. Two of the 99 healthy donors demonstrated a result of 1:160 speckled pattern by manual microscopy and NOVA View archived image.

3.4. Workflow Analysis. Although not the focus of our study, we investigated the impact of the NOVA View instrument in the laboratory workflow. We found that the system is a walk-away platform, with user friendly software, and the ability to interface with bar coded slides for positive patient identification.

4. Discussion

The recommendation of the ACR to use IIF as the preferred method has triggered the development and validation of automated systems for ANA determination. Although the ANA IIF test is the recommended method for ANA testing, the method has significant limitations, including a high degree of subjectivity [27]. With the availability of novel digital imaging systems, this limitation can be overcome [27]. However, careful evaluation and validation of those systems are required to ensure that the ANA results do not sacrifice clinical accuracy. One of the systems, the NOVA View, was evaluated in our study. In the beta software version 1.0.2 of NOVA View we found the instrument produced high quality images and excellent agreement with manual IIF testing. Properly comparing the archived NOVA View results to the manual results and the negative and positive sera, along with titer outcomes, demonstrated 97% concordance, in this study.

The NOVA View has a walk-away platform, user friendly software, and the ability to interface with bar coded slides for positive patient identification. In addition, the patient images are stored for later viewing without fluorescent burnout. However, the impact on the workflow might vary from laboratory to laboratory and needs to be quantified in further studies. The good agreement between interpretation using a microscope and using archived images on a screen holds promise to avoid the dark room, which is a source of transcription errors of results.

The specificity against healthy individuals in this study was in keeping with recent recommendations for the determination of anticellular antibodies [28]. However, the prevalence in SLE patients was somewhat lower than expected. This might be explained by the SLE population used and the relatively small cohort.

Since we did not analyze the performance of the NOVA View in terms of positive/negative discrimination and pattern recognition, further studies are needed. In recent years, several of those studies have already been performed [17–23, 25, 27, 29]. The internal LIU cutoff value causes the instrument to display the term "negative" for a sample that produces less than 100 LIU, whereas a positive result is displayed if the LIU is 100 or greater. The preset LIU cutoff of 100 does not always correlate between instrument generated outcome and manual microscopy on low positive/negative samples. The LIU cutoff can be adjusted to closely match the laboratory's manual reading during the validation process if desired. This does not change the image produced by NOVA View. Among many other systems, the NOVA View is an automated image recognition instrument.

Since this study, NOVA View has had two software updates. The current version, 1.0.3.1, contains a Single Well Titer (SWT) application that utilizes the LIU and assigns pattern to produce a calculated titer from one well. A recent study by Schouwers et al. concluding the estimation of fluorescent intensity offers clinically useful information and value added reporting [29].

Further studies are desired to underline the clinical utility of the NOVA View system in diagnostic specimens.

Conflict of Interests

The authors declare that there is no conflict of interests regarding the publication of this paper.

Acknowledgments

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Research Article

Anti-MDA5 Antibodies in a Large Mediterranean Population of Adults with Dermatomyositis

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A new myositis-specific autoantibody directed against melanoma differentiation-associated gene 5 (anti-MDA5) has been described in patients with dermatomyositis (DM). We report the clinical characteristics of patients with anti-MDA5 in a large Mediterranean cohort of DM patients from a single center, and analyze the feasibility of detecting this autoantibody in patient sera using new assays with commercially available recombinant MDA5. The study included 117 white adult patients with DM, 15 (13%) of them classified as clinically amyopathic dermatomyositis (CADM). Clinical manifestations were analyzed, with special focus on interstitial lung disease and its severity. Determination of anti-MDA5 antibodies was performed by a new ELISA and immunoblot technique. In sera, from 14 (12%) DM patients (8 CADM), MDA5 was recognized by ELISA, and confirmed by immunoblot. Eight of the 14 anti-MDA5-positive patients (57.14%) presented rapidly-progressive interstitial lung disease (RP-ILD) versus 3 of 103 anti-MDA5-negative patients (2.91%) ($P < 0.05$; OR: 44.4, 95% CI 9.3–212). The cumulative survival rate was significantly lower in anti-MDA5-positive patients than in the remainder of the series ($P < 0.05$). Patients with anti-MDA5-associated ILD presented significantly lower 70-month cumulative survival than antisynthetase-associated ILD patients. Among the cutaneous manifestations, only panniculitis was significantly associated with the presence of anti-MDA5 antibodies ($P < 0.05$; OR: 3.85, 95% CI 1.11–13.27). These findings support the reliability of using commercially available recombinant MDA5 for detecting anti-MDA5 antibodies and confirm the association of these antibodies with RP-ILD in a large series of Mediterranean patients with DM.

1. Introduction

In 2005, Sato et al. [1] identified a novel autoantibody recognizing a 140-kDa protein in patients with dermatomyositis (DM), particularly in those with clinically amyopathic dermatomyositis (CADM). The 140-kDa autoantigen, which was identified as melanoma differentiation-associated protein 5 (MDA5), is detected in 19% to 35% of the patients with DM. In the Asian population, this autoantibody seems to be associated with rapidly progressive interstitial lung disease and with severe cutaneous vasculopathy (skin ulceration, tender palmar papules, or both) [1–6]. Recently, the presence of anti-MDA5 antibody-associated dermatopulmonary syndrome was described in the white population [7–9].

MDA5, also known as interferon-induced helicase-1 (IFIH1), is a member of the retinoic acid-inducible gene I-like helicase (RIG-I or RLH) family of proteins, [10] which function by recognizing single-stranded RNA viruses and are involved in the innate immune response, including type I IFN production [11].

The main drawback to routine use of this antibody for clinical purposes is that its determination is limited to techniques that are only available in research laboratories, such as immunoprecipitation of radioactive-labeled protein [8] or enzyme-linked immunoassay (ELISA) using in-house fabricated recombinant proteins [12, 13].

Our objective was to evaluate the prevalence and clinical manifestations of anti-MDA5-positive patients in a large

cohort of DM patients from a single center in Barcelona, and to determine the feasibility of detecting this autoantibody with the use of more widely available techniques (ELISA and immunoblotting) with commercially available recombinant MDA5 as the antigen.

2. Patients and Methods

2.1. Patient Population. This study was performed in 117 adult patients (92 women) with DM (15 with clinically amyopathic DM). In addition, 45 patients with polymyositis (PM), 30 with systemic sclerosis (SSc), and 25 with systemic lupus erythematosus (SLE) were included as controls. Twenty-five healthy controls were also included to determine the cut-off value for establishing the positive status by ELISA. Healthy and disease controls were age and sex matched to the DM patients. The median age of DM patients was 52 years (range 22–81). The patients studied belong to a historical cohort diagnosed with idiopathic inflammatory myopathy at Vall d'Hebron General Hospital in Barcelona (Spain), between 1983 and 2012. Our center is a single teaching hospital with approximately 700 acute care beds, attending a population of nearly 450,000 inhabitants. All myositis patients in this population are referred to our hospital for diagnosis and therapy, regardless of the severity of the disease. Serum samples from these patients are routinely collected at diagnosis and during follow-up in our outpatient clinic, and stored at −80°C. Patients and controls included in the study gave informed consent for the use of their serum for research purposes. The study was approved by the institutional review board of our hospital.

The diagnosis of DM and PM was based on the criteria of Bohan and Peter [14, 15]. Only patients with definite or probable disease were included. The Sontheimer criteria were used to diagnose amyopathic DM [16]. Interstitial lung disease (ILD) was diagnosed according to the consensus classification of idiopathic interstitial pneumonias. The diagnosis of ILD was established by high-resolution CT findings, and rapidly progressive-interstitial lung disease (RP-ILD) was defined as a worsening of radiologic interstitial changes with progressive dyspnea and hypoxemia within 1 month after the onset of respiratory symptoms. [17] Cancer-associated myositis (CAM) was defined as cancer occurring within 3 years of the myositis diagnosis. Patients received treatment with corticosteroids and immunosuppressive drugs (methotrexate, azathioprine, calcineurin inhibitors [cyclosporine A or tacrolimus], or cyclophosphamide pulses) were added when needed; intravenous immunoglobulin was used as an adjuvant therapy, and biological therapy (rituximab) was also instituted when possible in refractory cases. A drug trial was defined as a single course from the beginning of the administration of a given drug to the time at which the drug was discontinued, or in the case of prednisone, as the time at which the dose was reduced to one quarter of the initial dose. Clinical data were obtained retrospectively by review of the patients' medical records.

2.2. Laboratory Tests and Serological Assay. Serum samples from each patient were screened by indirect immunofluorescence for antinuclear antibodies (ANA) using HEp-2 cells, and by a commercial ELISA used in our routine laboratory setting for antibodies against extractable nuclear antigens (Ro, La, RNP, Sm) and anti-histidyl-tRNA synthetase (anti-Jo-1). Anti-TIF1γ antibodies were detected by an in-house ELISA and confirmed by immunoblot [18]. In addition, all samples were tested by protein and RNA immunoprecipitation [19], which enabled detection of other synthetases and myositis-specific and myositis-associated antibodies (anti-Mi-2, anti-SRP, anti-Ro52, anti-Ro60, anti-La, anti-PM/Scl, anti-p155, and anti-U1RNP) that may have been overlooked by ELISA, and confirmed the ELISA results.

2.3. Anti-MDA5 ELISA. Briefly, 96-well ELISA plates (NUNC, Kamstrup, Denmark) were coated with 100 ng of purified recombinant MDA5 (OriGene, Rockville, MD), diluted in phosphate buffered saline (PBS), and left to stand overnight at 4°C. Wells were incubated for 1 hour at room temperature (RT) with blocking buffer (10% nonfat dry milk in PBS). Plates were then washed (HRP Wash, INOVA Diagnostic Inc., San Diego, CA); human serum samples diluted 1 : 100 in blocking buffer were added in triplicate: two to MDA5-coated wells and one to a PBS-coated well (without antigen) to determine the background absorbance. Plates were incubated at RT for 1 hour. After washing, HRP-labeled goat anti-human IgG antibody (INOVA Diagnostic Inc., San Diego, CA) was added to each well, and plates were incubated for 1 h at RT and washed again. Color development was performed with peroxidase reagent TMB Chromogen (INOVA Diagnostic Inc., San Diego, CA) and absorbances at 450 nm were determined. For each sample, the background absorbance from the PBS-coated well was subtracted from that of the corresponding MDA5-coated wells (the average of the two results). Sample absorbance was expressed as optical density units. The same positive serum (from patient 11, confirmed by IP by Casciola Rosen, from Baltimore, USA) was used as the reference in each assay.

2.4. Anti-MDA5 Immunoblot. Briefly, 5 μg of purified recombinant MDA5 (OriGene, Rockville, MD) was run on 4% to 12% polyacrylamide-SDS minigels with MOPS running buffer, and western blot was performed on a nitrocellulose membrane using the Invitrogen NuPAGE (Carlsbad, CA) electrophoresis system. [20] MDA5-transferred nitrocellulose was vertically cut into several strips and incubated for 1 hour at RT in PBS with 0.05% Tween (PBS-T) containing 3% nonfat dry milk (blocking buffer). Each strip was then incubated with the corresponding human serum sample diluted 1 : 100 in blocking buffer for 1 hour at RT. After washing, phosphatase alkaline-labeled goat anti-human IgG antibody (Dako, Glostrup, Denmark 1 : 2000) was added to each strip and strips were incubated for 1 hour at RT. Color development was performed by phosphatase reagent (BCIP/NBT, Sigma-Aldrich, St. Louis, MO). Based on signal intensity, the results were classified into negative, weak positive (+), or positive (++, +++) (Figure 1).

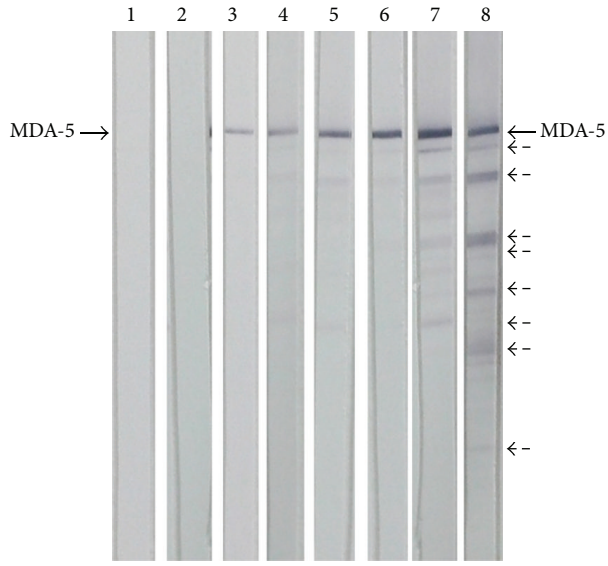

Figure 1: Immunoblots showing the reactivity of IgG antibodies from dermatomyositis patients against commercially available purified recombinant MDA5. Lanes 3 and 4 (+), 5 and 6 (++), and 7 and 8 (+++) were considered positive results. Lanes 1 and 2 corresponded to negative serum samples. Dashed arrows are probably degradation products of MDA5.

2.5. Statistical Analysis. Associations between anti-MDA5 antibodies and qualitative variables were evaluated with the chi-square and Fisher exact test. The strength of the associations between variables was measured using odds ratios (ORs) with 95% confidence intervals (CIs). The Mann-Whitney *U* test was used for comparisons of median values. The corresponding area under the curve (AUC) of the ROC analysis of anti-MDA5 antibody for detection of RP-ILD, CADM, and total DM was analyzed with 95% of CIs. All tests were two-sided, and probability (P) values of <0.05 were considered statistically significant. Cumulative survival rates were estimated by the Kaplan-Meier test. The log-rank test was also used to compare survival rates. All analyses were performed with SPSS, version 19.0 (SPSS, Chicago, IL).

3. Results

One-hundred and seventeen adult DM patients, 15 of whom had CADM, were included in the study. Anti-MDA5 was determined by our in-house ELISA and immunoblot techniques using a commercially available recombinant MDA5. The cut-off value for a positive result on ELISA was established at 0.188 absorbance units, which corresponded to 2 standard deviations above the mean value obtained for the 25 healthy controls. The other control subjects included 45 patients with polymyositis (PM), 30 with systemic sclerosis (SSc), and 25 with systemic lupus erythematosus (SLE) (Figure 2). Only two patients diagnosed with DM, 2 with PM, and 1 with SSc showed weak anti-MDA5 reactivity by ELISA, which was not confirmed by immunoblot; these results were considered false positives. Anti-MDA5 antibodies detected by ELISA and confirmed by immunoblot were only found in DM patients. ROC curve analysis for the positivity of anti-MDA5 of all patients with DM against controls and CADM patients versus remaining DM disclosed an AUC of 0.56, 95% CI 0.49–0.63 and 0.74, 95% CI 0.58–0.9, respectively. Patients with the highest absorbance unit values on ELISA also showed the strongest anti-MDA5 positivity on immunoblotting.

Fourteen patients, 8 with CADM, tested positive for anti-MDA5, which represents a prevalence of 12% of the DM patients from our cohort. Median (range) age at diagnosis of anti-MDA5 positive patients was 47 (28–60) years, which did not differ significantly from the remainder of the cohort. ANA was positive in 5 patients. Seven patients also tested positive to anti-Ro52, but none of them was positive for any antisynthetase antibody. Relevant clinical and immunological findings are summarized in Table 1.

3.1. Relationship between Anti-MDA5 and RP-ILD. Interstitial lung disease was present in 9 of the 14 (64.3%) patients with anti-MDA5 autoantibodies, and the condition was rapidly progressive in 8 patients. RP-ILD was more frequent in patients with CADM, both in the anti-MDA5 positive group (7 of 8 CADM versus 1 of 6 DM; $P < 0.05$) and in the overall cohort (8 of 15 CADM patients versus 3 of 102 DM patients; $P < 0.05$). When RP-ILD was evaluated in relation to anti-MDA5 positivity, a highly significant association was observed between the two parameters. Thus, 8 of the 14 anti-MDA5-positive patients presented RP-ILD versus 3 of the 103 anti-MDA5-negative patients ($P < 0.05$; OR: 44.4, 95% CI 9.3–212; AUC 0.84, 95% CI 0.68–1). Nevertheless, no association was found between anti-MDA5 ELISA titers at diagnosis of DM and development of a RP-ILD. Moreover, 6 of the 8 (75%) patients with anti-MDA5 and RP-ILD were Ro52-positive in comparison to only 1 of the 6 (16%) patients without RP-ILD.

Table 1: Clinical and immunological characteristics of our 14 MDA5-positive Mediterranean patients.

ID/ sex	Data onset Age (years)	Diagnostic	Dyspnea RP-ILD	Skin*	CT	ELISA MDA5	IB MDA5	Other antibodies	Highest CK levels (IU/L)^	Cancer	ICU	Lung pathology	LT	Exitus/ follow-up
1/F	April 2010 54	CADM	April 2010 Yes	—	NSIP	1.270	++	ANA (−) Ro52 (+) RF (+)	—	No	No	NA	No#	No June 2012
2/F	February 2005 57	DM	No	MH Panniculitis	Normal	1.637	+++	ANA 1/320	1856	Breast March 2007	No	—	No	No October 2012
3/M	June 2006 46	DM	February 2007 Yes	MH Ulcers	Ground glass	0.386	+	ANA (−) Ro52 (+)	4437	No	No	NA	No	No April 2012
4/F	November 1993 41	DM	April 2000 No	Panniculitis Calcinosis	Ground glass	1.343	+++	ANA (−)	304	No	No	NA	No	No October 2012
5†/F	March 2000 53	CADM	October 2000 Yes	Ulcers Panniculitis	Alveolar infiltrates	2.744	+++	ANA 1/640, Ro52 (+) RF (+)	—	No	November 2000	DAD§	Yes November 2000	Yes November 2000
6†/F	June 1992 28	CADM	August 1992 Yes	—	Alveolar infiltrates	1.220	++	ANA (−) Ro52 (+) RF (+)	—	No	September 1992	DAD§	No‡	Yes September 1992
7†/M	January 2000 69	CADM	May 2000 Yes	Ulcers	Ground glass	2.999	+++	ANA 1/160 U1RNP (+)	—	Lung March 2000	—	NA	No	Yes September 2000
8/F	February 1996 38	DM	No	—	Normal	1.340	+++	ANA 1/160 TIF1γ (+)	583	Ovarian July 1996	No	No	No	Yes December 1998
9/F	July 2004 30	CADM	No	Panniculitis Calcinosis	Normal	0.913	++	ANA (−)	—	No	No	No	No	No October 2012
10/M	May 1992 55	DM	No	Ulcers	Normal	2.251	+++	ANA 1/640 Ro52 (+)	136	Lung March 1996	—	No	No	Yes September 1997
11/M	June 2012 54	CADM	June 2012 Yes	—	Alveolar infiltrates	2.054	+++	ANA (−) Ro52 (+)	—	No	October 2012	DAD§	No#	Yes October 2012
12†/M	February 2000 46	CADM	February 2000 Yes	MH	Lung fibrosis	0.926	++	ANA (−)	—	No	November 2012	DAD§	Yes May 2000	Yes March 2004
13/F	March 2012 53	CADM	May 2012 Yes	MH	Alveolar infiltrates	2.456	+++	ANA (−) Ro52 (+)	—	No	No	NA	No#	Yes December 2012
14/F	July 2010 52	DM	No	Panniculitis	Normal	1.208	++	ANA (−)	550	No	No	No	No	No June 2012

ANA: antinuclear antibodies; CADM: clinically amyopathic dermatomyositis; DAD: diffuse alveolar damage; DM: dermatomyositis; F: female; ICU: intensive care unit; M: male; MH: mechanic's hands; NA: not available; NSIP: nonspecific interstitial pneumonia; RF: rheumatoid factor; RP-ILD: rapidly progressive interstitial lung disease. #Proposed for lung transplantation (LT), but expired before it was available or improved and it was not necessary. †Previously reported in [25]. §Necropsy or lung explantation. ‡Not available in 1992. Patients 8 and 10 died from cancer and DM activity, respectively, and the remaining deceased patients died from acute respiratory failure. MDA5 value by ELISA is expressed in absorbance units. *All patients presented with classical skin manifestations (i.e., Gottron papules, heliotrope rash). The other skin manifestations reported in Table 1 are referred to nonclassic cutaneous involvement, and both (classic or not) are referred to the moment when dermatomyositis was diagnosed. ^Creatine Kinase (CK). Normal value levels of CK (<195 IU/L).

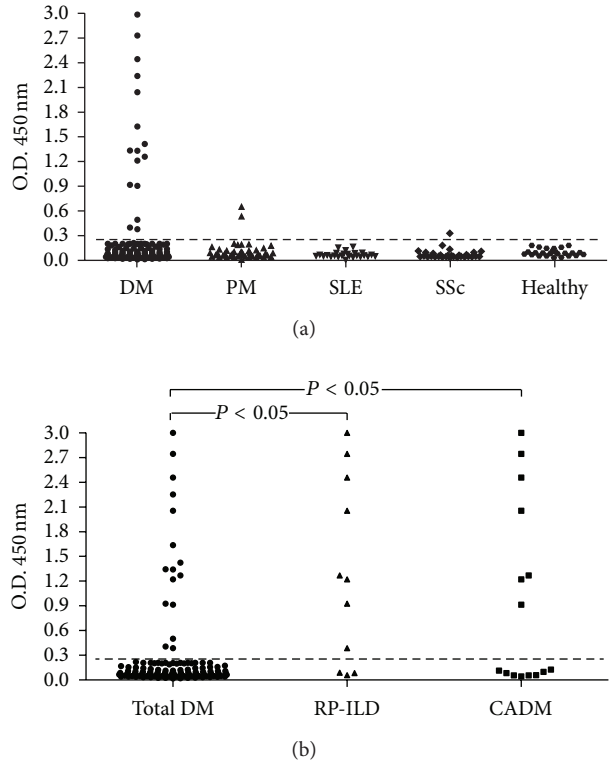

Figure 2: Representation of the anti-MDA5 ELISA test results in patients with dermatomyositis (DM) ($n = 117$) and controls groups: polymyositis (PM) ($n = 45$), systemic sclerosis (SSc) ($n = 30$), systemic lupus erythematosus (SLE) ($n = 25$), and healthy controls ($n = 25$). Panel (b) shows anti-MDA5 ELISA of patients with DM ($n = 117$) and individual subgroups of DM patients: rapidly progressive-interstitial lung disease (RP-ILD) ($n = 11$) and clinically amyopathic dermatomyositis (CADM) ($n = 15$). The cut-off value for a positive result was established at 0.188 absorbance units which corresponded to 2 standard deviations above the mean value obtained for the 25 healthy controls (dashed line).

3.2. Relationship between Anti-MDA5 and Cutaneous Manifestations and Cancer. The CADM diagnosis was significantly associated with anti-MDA5 positive status (8 of 14 MDA5-positive patients versus 7 of 103 MDA5-negative ($P < 0.05$; OR: 18.3, 95% CI 4.9–67.6)). No differences were observed in the frequency of Gottron's papules, heliotrope rash, photosensitivity, shawl or "V" sign, cuticular overgrowth, calcinosis, or the presence of mechanic's hands when the anti-MDA5-positive and anti-MDA5-negative groups were compared. Panniculitis was the only manifestation significantly associated with the presence of anti-MDA5 (5 out of 14 anti-MDA5-positive versus 13 out of 103 anti-MDA5-negative; $P < 0.05$; OR: 3.85, 95% CI 1.11–13.27); nevertheless, a multivariate analysis was not possible to be performed due to methodological reasons.

Cancer was diagnosed in 4 out of 14 (28.6%) patients with anti-MDA5 autoantibodies, and 3 (21%) of them fulfilled criteria of CAM. However, no association was found between anti-MDA5 autoantibodies and CAM ($P > 0.05$). A similar result was obtained when we repeated the analysis after excluding from the cohort the anti-TIF1γ-positive patients (28 patients) in order to avoid a possible confounding effect of the presence of these patients in the control group.

3.3. Survival Rates of Anti-MDA5-Positive Patients. The cumulative 70-month survival rate was significantly lower (38%) in the group of patients with anti-MDA5 than in the remainder of the cohort (62%) (log-rank test, $P < 0.05$) (Figure 3(a)). Comparison of cumulative 70-month survival between anti-MDA5-associated ILD and antisynthetase-associated ILD also showed a statistical difference (log-rank test, $P < 0.05$) (Figure 3(b)). No differences in 70-month survival were observed between the CADM group and the classic DM group in anti-MDA5- positive patients. All groups were comparable in age, gender, and number of immunosuppressive agents added to the corticosteroid treatment. Differences in survival could not be attributed to a higher proportion of "deaths directly related to cancer" between groups.

4. Discussion

The results of this study prove the feasibility of detecting antibodies against MDA5 in adult patients with DM by in-house ELISA and immunoblot techniques using commercially available recombinant MDA5 as the antigen. In addition, the findings in our patients contribute to support the previously reported association of anti-MDA5 antibody

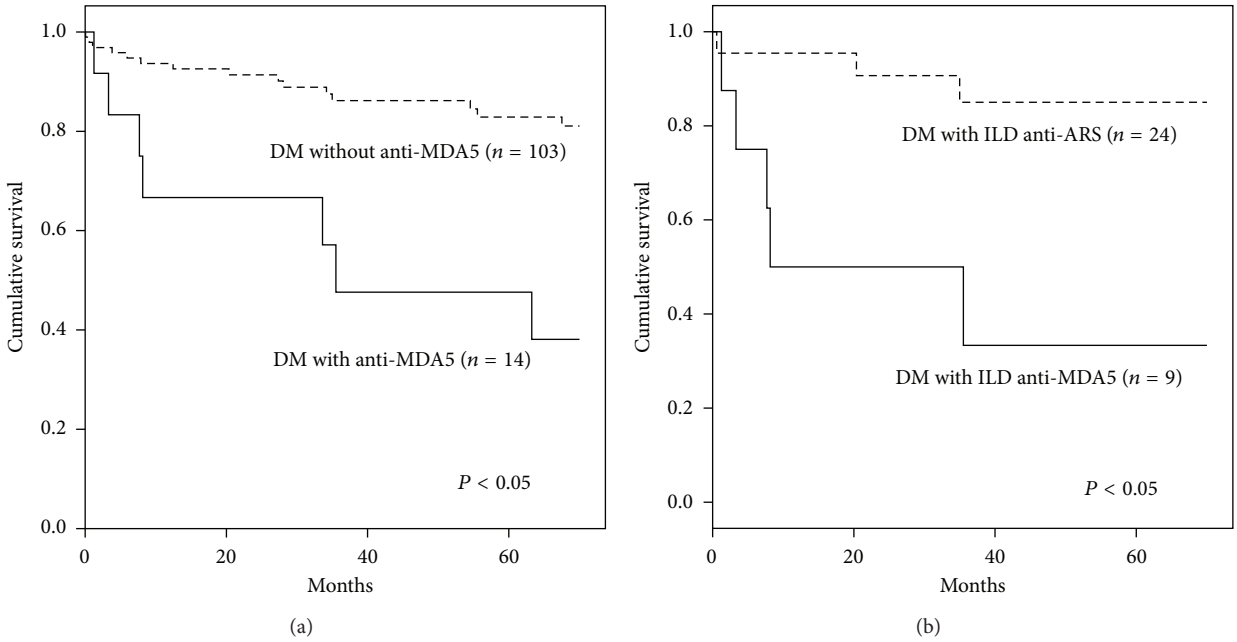

Figure 3: Cumulative 70-month survival rates for DM patients with and without anti-MDA5 antibody (a) and for DM patients with ILD associated with anti-ARS or anti-MDA5 (b). The 70-month cumulative survival rates were calculated using the Kaplan-Meier test. The log-rank test was also used to compare survival rates. ARS: aminoacyl-tRNA synthetase; DM: dermatomyositis; ILD: interstitial lung disease.

with RP-ILD and CADM. This association has been mainly described in Asian patients. [1–6], and the only studies performed in a white population have come from the United States [7–9]. Our results in a large series of Mediterranean patients from a single reference center, together with those from other articles published on this topic, [1–8] indicate that anti-MDA5 antibodies may be a hallmark of adult CADM patients with RP-ILD regardless of their origin.

Furthermore, some authors have suggested an association between this autoantibody and a specific, severe skin vasculopathy in adult DM, characterized by vascular fibrin deposition with variable perivascular inflammation [3, 7, 8]. We found an association only with panniculitis, one of the mucocutaneous findings previously described by Fiorentino et al. [8] in white adults with DM and anti-MDA5 antibody.

Although we routinely test all serum samples from patients with inflammatory myopathies by immunoprecipitation assays using ^{35}S-protein-labeled HeLa cells, until the development of our proposed method, we had not been able to clearly identify patients with anti-140 kDa antibodies (anti-CADM-140; i.e, anti-MDA5). In our analyses, this polypeptide migrated to an area in which almost 70% of sera, including those from normal subjects, immunoprecipitated a weak line around 140 kDa. Hence, until anti-MDA5 was described by Sato et al. [1, 6], and we tested these patients with a commercially available recombinant MDA5 by our in-house ELISA and immunoblot techniques, we were unable to recognize this autoantibody. Our positive patients correspond to a period of 30 years of follow-up. Our first patient with CADM and RP-ILD died nearly 20 years ago, and anti-MDA5 was detected in a stored frozen serum sample by our in-house techniques. Thus, these methods could represent a significant advancement in identification of this autoantibody, even in laboratories with standard equipment.

Anti-Ro52 antibodies were present in most of our patients with RP-ILD and anti-MDA5, an association that has only recently been reported in anti-MDA5-positive ILD patients [9]. This fact confers relevant significance on anti-Ro52 as a costimulatory autoantibody, a concept reported in patients with antisynthetase syndrome [21, 22].

One patient was positive to both anti-MDA5 and anti-TIF1γ. To our knowledge, this is the first description of this association. Coexistence of two different myositis-specific antibodies has been rarely reported [19, 23]. Hence, the situation of this patient is intriguing and warrants further investigation.

The clinical course of anti-MDA5-positive DM patients can be divided into three groups. First (and most important from the prognostic perspective), is the group of patients with CADM and RP-ILD, who usually have a poor prognosis and a mortality rate of nearly 50% despite aggressive immunosuppressive therapy and even lung transplantation. Second, the group with CADM and little lung involvement, who show skin manifestations, such as ulcerations, palmar pustules, and perhaps panniculitis, as was reported here. The prognosis does not seem to be unfavorable in this group. And finally, the third group of patients, who have ILD that is not rapidly progressive and shows a disease pattern similar to that of classic antisynthetase syndrome. [9] Differences in the prognosis between the first and the third group may be due to genetic background, and either early immunosuppressive therapy or use of certain drugs, such as calcineurin inhibitors [24].

The existence of a clinical syndrome of rapidly progressive ILD (usually in antisynthetase-negative patients) was recognized some years ago [25], but the absence of useful biomarkers made it difficult to characterize these patients. The discovery of anti-MDA5 antibodies will help to better define this population, facilitate an early diagnosis, establish the prognosis, and ultimately enable the development of randomized clinical trials to determine the optimal therapy in anti-MDA5-positive patients with a poor prognosis. Moreover, as it has been recently described, anti-MDA5 antibody measurement seems to be useful for monitoring disease activity [13, 26].

As our results show, ELISA confirmed by immunoblot with commercially available recombinant MDA5 antigen are useful techniques for anti-MDA5 detection that can be reliably performed in a standard laboratory setting, with potential application in clinical practice.

Conflict of Interests

This is an original work, and all authors meet the criteria for authorship, including acceptance of responsibility for the scientific content of the paper. There is no conflict of interests.

Authors' Contribution

Moises Labrador-Horrillo (guarantor), and Albert Selva-O'Callaghan had the original idea, and together with Ernesto Trallero-Araguas, designed the study. Moises Labrador-Horrillo, Maria Angeles Martinez, Eva Balada, and Cándido Juárez, performed the laboratory techniques (ELISA, immunoblot and immunoprecipitation). Albert Selva-O'Callaghan, Ernesto Trallero-Araguas, and Miquel Vilardell-Tarres, managed the patients with dermatomyositis. All authors contributed to, and approved, the final paper.

Acknowledgment

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Research Article

Atrioventricular Conduction Delay in the Second Trimester Measured by Fetal Magnetocardiography

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Introduction. Fetal AV block in SSA/Ro pregnancies is generally not seen before 18-week gestation and onset is rare after 28-week gestation. If complete AV block appears, it is believed to be irreversible. The purpose of the study was to evaluate precise electrophysiological AV conduction from 18-week gestation onwards. *Patients and Methods*. 21 fetuses of pregnant women with collagen vascular diseases were included in the study group and 59 healthy fetuses served as controls. In addition to fetal echocardiography, fetal magnetocardiography (fMCG) was used to investigate precise electrophysiological fetal cardiac time intervals (fCTIs). *Results*. The PR segment (isoelectric segment between the end of the P wave and the start of the QRS complex) was significantly prolonged ($P < 0.036$ 2nd trimester, $P < 0.023$ 3rd trimester) in both trimesters within the study group. In fetuses less than 23-week gestational age, a nearly complete separation was found, where a PR segment of 60 ms or greater completely excluded control fetuses. All other fCTIs did not differ significantly. None of the fetuses progressed to a more advanced heart block. *Conclusion*. Slight antibody effects in pregnancy, leading to PR segment prolongation, can already be seen from 18-week gestation onwards by fMCG. Serial fetal Doppler echocardiography and additional fMCG can be useful methods to measure early and precise AV conduction time, to achieve best surveillance for these high-risk pregnancies.

1. Introduction

Substantial morbidity and mortality of fetuses in patients with anti-SSA/Ro antibodies in pregnancy are associated with the development of congenital heart block [1–4]. Fetal AV block in SSA/Ro pregnancies is generally not seen before 18-week gestation and onset is rare after 28-week gestation [5]. If complete congenital heart block in these fetuses occurs, it is believed to be irreversible. Nevertheless, intrauterine therapy might be possible, although it is empiric at the moment. The rationale for treatment strategies is to identify the heart block as early as possible and to diminish the inflammatory insult to the heart by lowering the maternal antibodies [6]. Immune-mediated AV block may benefit from in utero treatment with fluorinated steroids, IVIG, or both. Dexamethasone is believed to reduce inflammation [7–9]. Although no clear consensus exists, most clinicians use dexamethasone 4–8 mg/day to treat not only second-degree AV block and recent onset AV block but also severe cardiac dysfunction and hydrops.

Several investigators have reported a transient prolongation of AV conduction time by echocardiography during midtrimester, which was still present on postnatal electrocardiograms (ECG) in 50% of the subjects. The long-term prognosis in these studies was reported as being excellent [10, 11]. These findings might indicate a time frame, where reversal of incomplete block without treatment can be seen.

The methods used are mainly Doppler techniques which measure the mechanical rather than the electrophysiological

events to obtain AV intervals. Fetal magnetocardiography (fMCG) might fill in this gap. This innovative method is more precise in detecting fetal conduction and arrhythmias [12–14]. However, fMCG generally captures each of the cardiac time intervals (P wave, QRS complex, and T waves, RR-, PR-, and QT intervals) in fetuses over 24-week gestation; in fetuses below 24-week gestation, which is the most important time frame in the development of AV conduction delay, only QRS and RR intervals can be reliably measured in most fetuses [15, 16]. In consequence, a new analyzing method was investigated. We have previously reported PR segment prolongation, relative to controls, in sixteen 3rd trimester fetuses [17], however, analysis of fMCG intervals at younger gestations was difficult until a new method of signal extraction based on a combination of orthogonal projection and independent component analysis was developed. With the help of this new method, measurements of fCTIs were possible from 18-week gestation onwards [17]. The aim of this study was to investigate precise electrophysiological fetal cardiac time intervals in these high-risk fetuses from 18-week gestation onwards by fMCG. This might help to understand the pathophysiology of reverse AV prolongation.

2. Patients and Methods

2.1. Patient Population. Baseline characteristics of all 80 patients for this observational study were evaluated with regard to medical history, previous pregnancy outcomes, and medication intake.

21 fetuses of pregnant women with collagen vascular diseases such as systemic lupus erythematosus or Sjogren's syndrome were included in the study group.

At study entry, all patients of the study group fulfilled the following inclusion criteria: presence of anti-SSA/Ro and/or anti-SSB/La antibodies tested by an enzyme linked immunosorbent assay (ELISA) and/or an immunofluorescence test, an immunodiffusion test, and dot blots by a commercial laboratory. Rheumatologic disease was diagnosed by a rheumatologist according to defined criteria [18, 19]. There was no limit concerning the duration of medication intake. Pregnancies over 18-week of gestation with a normal heart beat and a structural normal heart were included.

Exclusion criteria for all neonates were chromosomal abnormalities, malformations, and congenital infections.

Data of the study group were compared to already established norm values of healthy women with uncomplicated pregnancies and normally developing fetuses. Neonatal outcomes defined as normal AV conduction by physical exam and established normative cardiac time intervals for age were assessed by a paediatrician by clinical routine examination, fetal heart rate monitoring, and neonatal ECG.

The study was approved by the ethics review board of the University Hospital Tuebingen. Informed written consent was obtained from each subject.

2.2. Methods. At the beginning of the study, conventional echocardiography was performed in the study group to evaluate structural cardiac abnormalities, myocardial function, and fetal heart rate, in addition to regular ultrasound examinations.

fMCG measurements were performed in the study group and in the control group.

Prior to the beginning of each fMCG measurement, ultrasound was performed in all patients to check the fetal position and to localize the fetal heart. Furthermore, cardiotocography was performed over a 20-minute period to obtain complete information about the health of the fetus.

2.2.1. Data Acquisition. The fMCG recordings were acquired using a 156-channel biomagnetic system with channels arranged in a curved array that matched the shape of the gravid abdomen (SARA system, VSM Med Tech Ltd., Port Coquitlam, Canada). All of the measurements were recorded with a sampling rate of 1220.7 Hz in a magnetically shielded room (Vakuumschmelze, Hanau, Germany). The length of the recordings ranged from nine to 35 minutes. Data collection was performed between 18 and 38 weeks gestation, focusing primarily on second trimester.

2.2.2. Data Processing. The analysis of the fetal heart signals was performed according to our previously published work [17]. An automated algorithm using orthogonal projection and independent component analysis was applied to reconstruct the fetal heart signal [20–23]. fCTI evaluation was performed using a custom-made MATLAB program (R2008b, Mathworks, Natick, MA, USA).

The time points identified were used to calculate the duration of the CTI as follows.

P wave = $P_{end} - P_{onset}$, QRS complex = $QRS_{end} - QRS_{onset}$. The QT interval was defined as $T_{end} - QRS_{onset}$. The PR interval was determined as P wave + PR segment, whereas PR segment was defined as P_{end} to QRS_{onset}. The PR segment may more accurately reflect the AV conduction as the PR interval, because it eliminates any intra-atrial conduction delay reflected by the P wave duration measurement. All fMCG recordings were reviewed by at least one physician who has extended experience in pediatric cardiology.

2.3. Statistical Analysis. Statistical analysis was performed using SPSS 20.0 (IBM) for Windows. Normal distribution was tested using Kolmogorov-Smirnov Test. ANCOVA was used for data analysis and the influence of age, gender, and birth weight was tested. $P < 0.05$ was regarded as statistically significant.

3. Results

3.1. Patient Population

3.1.1. Study Population. 21 mothers were included in the study group. The median age of the mothers with systemic lupus erythematosus ($n = 15$ patients) or Sjogren's syndrome ($n = 6$ patients) was 31 years (range 21–46 years) (Table 1). The maternal suppressive therapies in these 21 patients were low-dose prednisolone ($n = 13$), high-dose prednisolone ($n = 1$),

Table 1: Baseline characteristics of the mothers, fetuses, and newborns.

	Study group (n = 21 patients)	Control group (n = 59 patients)
Mother		
Median age of mothers (years; range)	31 (21–46)	33 (25–50)
Prednisolone ≤10 mg (n)	13	1
Prednisolone >10 mg (n)	1	0
Hydroxychloroquine (n)	12	0
Azathioprine (n)	6	0
Cyclosporine (n)	1	0
Thyroid medication (n)	3	12
Aspirin (n)	9	1
Fetus		
Median age of fetuses measured in 2nd trimester (weeks; range)	23 (18–26)	24 (19–26)
Median age of fetuses measured in 3rd trimester (weeks; range)	30 (27–38)	33 (28–38)
Newborn		
Male newborns (n)	9	34
Female newborns (n)	12	25
Preterm >32 and <37 weeks of gestation (n)	6	4
Mean birth weight (g; SD)	2845 ± 530	3470 ± 480
Mean birth length (cm; SD)	49 ± 3	51 ± 2
Small for gestational age (n)	6	1

hydroxychloroquine (n = 12), cyclosporine (n = 1), and azathioprine (n = 6). Most of the patients received more than one medication.

21 fetuses were measured with a median gestational age of 28 weeks (range 18–38 weeks). Six neonates were too small for gestational age.

3.1.2. Control Population. 59 pregnant women were included in the control group. Mean age of the women was 33 years (range 25–50 years). All women were healthy except for one with gestational diabetes. Chronic diseases were found in the following patients: thalassaemia minor (two patients), Crohn's disease (one patient), and factor V Leiden mutation (one patient). Twelve women had a previous history of hypothyroidism but were euthyroid at time of measurement.

59 fetuses were measured with a median gestational age of 32 weeks (range 19–38 weeks). Neonatal outcome revealed 59 healthy newborns. Four fetuses were born prematurely (>32 and <37 weeks of gestation). One newborn was small for gestational age whereas three newborns were large for gestational age.

3.2. Fetal Cardiac Time Intervals. Altogether 36 measurements in 21 patients were included in the study group and 63 measurements in 59 subjects were included in the control group. The measurements included measurements >24 weeks gestation of the previous study.

The fCTIs for all patients are shown in Tables 2 and 3. Table 2 focuses on second trimester (18–26 weeks of gestation), whereas Table 3 has its impact on late gestational ages (27–38 weeks of gestation). As the mean birth weight (shown in Table 1) was significantly smaller (P = 0.013) in the study group compared to the control group, P values were adjusted for this parameter. In addition, P values were adjusted for age and gender.

Table 2: Cardiac time intervals of the study group compared to those of the control group at 18–26 weeks of gestation (P value is adjusted for gestational age, gender, and weight).

Characteristics	Study group n = 18 measurements mean ± SD (ms)	Control group n = 19 measurements mean ± SD (ms)	Statistical significance (ANCOVA)
P wave	38 ± 7	43 ± 6	ns
PR segment	58 ± 11	48 ± 12	P = 0.036
PR interval	95 ± 14	91 ± 13	ns
QRS complex	51 ± 12	53 ± 7	ns
T wave	133 ± 37	157 ± 46[#]	ns
QT interval	240 ± 40	259 ± 40[#]	ns

[#]n = 16 measurements due to low identification rate.

The PR segment (isoelectric segment between the end of the P wave and the start of the QRS complex) was significantly prolonged in both trimesters within the study group (P < 0.036 2nd trimester, P < 0.023 3rd trimester). All other CTIs did not differ significantly. Available postnatal ECGs in the study group did not show first-, second- or third-degree AV block.

Table 3: Cardiac time intervals of the study group compared to those of the control group at 27–38 weeks of gestation (P value is adjusted for gestational age, gender, and weight).

Characteristics	Study group $n = 18$ measurements mean ± SD (ms)	Control group $n = 44$ measurements mean ± SD (ms)	Statistical significance (ANCOVA)
P wave	48 ± 10	55 ± 13	ns
PR segment	58 ± 11	51 ± 13	$P = 0.023$
PR interval	106 ± 15	104 ± 20	ns
QRS complex	56 ± 5	57 ± 7	ns
T wave	121 ± 13	128 ± 31[#]	ns
QT interval	250 ± 36	250 ± 42[#]	ns

[#]$n = 38$ measurements due to low identification rate.

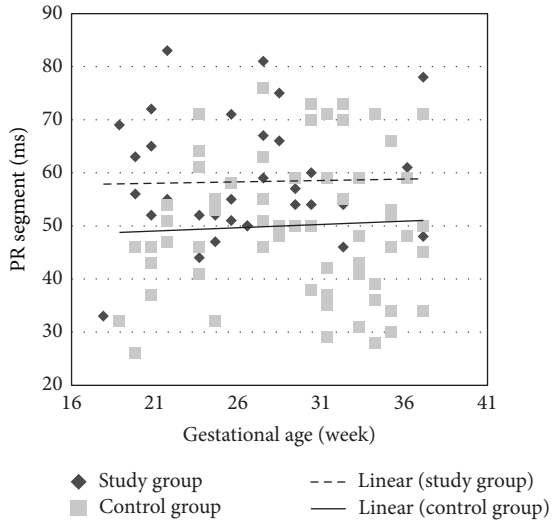

Figure 1: PR segments of study group and control group. Lines indicate 50th %ile measurements.

4. Discussion

The main finding in this study was that the PR segment (PR interval − P wave), measured by fMCG, was significantly prolonged in the study group not only in the third but also in the second trimester when compared to controls. Four of the study subjects and only one of the control subjects had PR segment measurements equal to or exceeding 75 ms. In fetuses less than 23-week gestational age, a nearly complete separation was found, where a PR segment of 60 ms or greater completely excluded control fetuses (see Figure 1). All other fCTIs did not differ significantly and none of the fetuses progressed to second- or third-degree heart block.

These findings support the concept that mild AV conduction delays may not progress. In a large multicenter study, first-degree AV block did not predict development of advanced AV block, whereas tricuspid regurgitation of moderate or severe degree, and endocardial fibroelastosis, was associated with subsequent onset of 2nd or 3rd degree AV block.

Van Leeuwen and colleagues and Stinstra and colleagues, both, have reported dependency of fCTIs (also the PR segment duration) on gestational age and gender [16, 24, 25]. Additionally, subjects in the study group had a lower weight at birth than those of the control group [26]. However, significant PR segment prolongation was found in the study group, relative to the control group, even after adjusting for all these possible confounding factors.

The new analyzing method improved signal detection and reconstruction in early gestational ages. In consequence, a total of 99 measurements (in 80 patients) could be achieved, 18 under 24-week gestational age. In this study, PR segment prolongation was additionally found in the second trimester (from 18-week gestation onwards) by fMCG. None of the fetuses developed AV block. The prolongation of the PR segment duration might indicate antibody effects already present in the second trimester of pregnancy.

Jaeggi and colleagues reported similar findings by echocardiography from 15 untreated fetuses either with AV prolongation between 2 and 6 z-scores or with type one second-degree block. None of the fetuses developed progressive heart block [27].

In addition Sonesson and colleagues reported eight of 24 fetuses who had signs of first-degree block in their study. These AV blocks, measured by Doppler echocardiography, mainly reverted spontaneously. One of these fetuses had progression to complete block, another showed recovery from second-degree to first-degree block with treatment [28]. The lack of progression of first-degree AV block to more severe block makes treatment on the basis of first-degree AV block unnecessary, except perhaps in the most severe prolongation. Rein and colleagues have reported treating patients with dexamethasone for first-degree AV block and attributed the lack of progression as a sign that steroids were effective [29].

Mechanical measurements by Doppler echocardiography and electrophysiological measurements by fMCG, both, show AV prolongation. Stable but also progressive AV prolongation was described in different studies. Therefore serial fetal Doppler echocardiography and additional fMCG measurements of AV time intervals are proposed as a useful method to measure early and precise AV conduction time to achieve best surveillance of these high-risk pregnancies.

In summary, antibody in pregnancy patients might have an effect on the fetal atrioventricular conduction already from early second trimester onwards. But this effect can be reversible and does not consecutively lead to congenital heart block.

To develop new management strategies, fMCG measurements in addition to Doppler echocardiography might be helpful. However, a higher number of these high-risk patients in multicenter studies of this rare condition are probably necessary.

Conflict of Interests

The authors declare that there is no conflict of interests.

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Review Article

A New ELISA for Dermatomyositis Autoantibodies: Rapid Introduction of Autoantigen cDNA to Recombinant Assays for Autoantibody Measurement

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Advances in immunology, biochemistry, and molecular biology have enabled the development of a number of assays for measuring autoantibodies. ELISA has been widely used, because it can deal with relatively large numbers of serum samples more quickly than other immunologic methods, such as immunoblotting and immunoprecipitation. Recombinant autoantigens, which are generally produced in *E. coli* using the relevant cloned cDNA, are necessary for ELISA. Conventional clinical ELISA tests are limited in their ability to purify proteins free of bacterial contaminants, and the process is labor intensive. We recently developed new ELISA tests that utilize simple *in vitro* transcription and translation labeling of autoantigens in order to measure dermatomyositis- (DM-) specific autoantibodies, including autoantibodies to Mi-2, MDA5, NXP-2, TIF1-α, and TIF1-γ. This method may allow for the rapid conversion of cDNAs to a chemiluminescent ELISA to detect autoantibodies that are found not only in DM but also in other autoimmune diseases.

1. Introduction

Idiopathic inflammatory myopathies (IIMs) are a group of systemic autoimmune diseases that include polymyositis (PM), dermatomyositis (DM), and inclusion body myopathies. Several myositis-specific autoantibodies (MSAs) are associated with certain clinical forms of IIM, and they are useful tools for predicting the prognosis [1]. For example, anti-MDA5 antibody-positive patients demonstrate rapidly progressive interstitial lung disease (ILD), and anti-TIF1-γ antibody-positive patients are often complicated with cancer. In contrast, anti-Mi-2 antibodies are a serological marker for favorable prognosis in patients with classical DM who present with typical cutaneous manifestation and myositis. Autoantibodies to TIF1-γ are also present in juvenile DM as well as anti-MJ antibodies, and the latter recognize with NXP-2. Autoantibodies in DM tend to be mutually exclusive, thus enabling specific immune responses to differentiate between clinical subsets. It was recently clarified that anti-p155/140 antibodies, which were originally named for the molecular weight of the antigens [2], react to TIF1-γ and TIF1-α, respectively [3]. It is an exception that anti-TIF1-α antibodies appear with two mutually different prognostic markers: anti-TIF1-γ antibodies and also anti-Mi-2 antibodies [4].

Laboratories have been using several methods for detecting various autoantibodies: indirect immunofluorescence, immunoprecipitation (IPP), Western blotting (WB), and enzyme-linked immunosorbent assay (ELISA). ELISA-based serologic screening is highly sensitive and efficient, but it requires highly purified recombinant protein. The efficiencies of protein expression, purification, and stability limit the development of a novel ELISA and increase the risk of false-positive antibody detection. At present, many purified recombinant proteins are commercially available; however, full-length recombinant autoantigens are not always available. Moreover, even when they are available, their prices are often very high. Recently, we have developed an ELISA for the

detection of antibodies in sera with biotinylated recombinant protein by *in vitro* translation and transcription (TnT) and have detected DM-specific autoantibodies in our DM cohort [4–6]. This review introduces our newly developed ELISA tests, which use recombinant autoantigens to measure DM-specific autoantibodies, mainly autoantibodies to Mi-2, and clarifies the clinical significance of the new assay. This method may allow for the rapid conversion of cDNAs to a chemiluminescent ELISA in order to detect autoantibodies not only in DM but also in other autoimmune diseases.

2. ELISA with Commercially Available or In-House Prepared Recombinant DM Autoantigens

Recent works have clarified new DM-specific autoantigens, MDA5, TIF1-$\alpha/\beta/\gamma$, NXP2, and SAE [1]. In some recent studies, ELISAs with some of these commercially available or in-house prepared recombinant autoantigens were used. An ELISA measuring anti-MDA5 antibodies has been used in some works [7–9]. cDNA of MDA5 was cloned by immunoscreening with a patient's sera, and its recombinant protein produced by a baculovirus expression system was used for an ELISA [7]. The analytical sensitivity and specificity of this anti-MDA5 antibody ELISA were 85% and 100%, respectively. Anti-MDA-5 antibody levels measured by this ELISA closely correlated with the severity of skin ulcerations, ILD, and the prognosis of the disease in a Chinese study [8]. In a Japanese study, the median value of the anti-MDA5 antibody titer on admission was higher in patients who later died than in those who survived [9]. The decline index of the anti-MDA5 antibody titer after treatment was lower in the subset of patients who died than in the subset of patients who lived. Sustained high levels of anti-MDA5 antibody were present in the patients who died. In light of these results, anti-MDA5 antibody ELISA is useful for evaluating the response to treatment and the status of ILD in patients with anti-MAD5 antibody-positive DM.

Fujimoto et al. used an ELISA with commercially available recombinant TIF1γ and TIF1α to investigate longitudinal changes in serum antibody titers [3]. After treatment, the titer of anti-TIF-1γ antibodies decreased in all 8 patients, while the titer of anti-TIF-1α antibodies did not always decrease. The pathological significance of the titers of TIF1-γ/α needs further investigation.

Satoh et al. used commercially available recombinant TIF1-$\alpha/\beta/\gamma$ in an ELISA [11]. They confirmed the presence of these autoantibodies by using IPP-WB, antigen-capture ELISA, and ELISA with recombinants. The results of the ELISA with recombinants were consistent with the results shown by other immunological methods.

We also tried to perform an ELISA using commercially available recombinant SAE1 [12]. Anti-SAE antibodies were screened for 110 patients with DM, and 2 patients were found to have anti-SAE antibodies. Although anti-SAE autoantibodies also react to another subunit, SAE2 [13], an ELISA with recombinant SAE2 protein has not been reported.

3. Recombinant Protein Produced by *In Vitro* Translation and Transcription

Many studies have investigated autoantibodies by using recombinant protein produced by TnT. For example, in studies on cDNA cloning of autoantigens, this eukaryotic expression system, which often uses rabbit reticulocyte lysate, has been utilized in order to confirm whether patient's sera react to candidate clone's product and whether the clone product's mobility on SDS-PAGE is the same as the mobility of the endogenous cellular antigen [14–16]. Recombinant proteins produced by TnT are generally labeled with ^{35}S-methionine. The productive efficiency is theoretically influenced by the presence of the Kozak's consensus sequence around the AUG initiation codon and the numbers of methionine residues. Recent commercial kits for TnT contain all the necessary materials, except for highly purified DNA, to produce recombinants. The recombinant protein can be used for IPP without any pretreatment, since it is generally produced in soluble form.

To eliminate the need for radioactive materials, commercial products for biotin-labeled recombinants are also available. This labeling utilizes precharged *E. coli* lysine tRNAs, which are chemically biotinylated at the ε-aminogroup. Biotinylated proteins can be detected by the binding of streptavidin-alkaline phosphatase or streptavidin-horseradish peroxidase using a colorimetric or chemiluminescent detection system. Although the presence of biotinylated lysines may affect the antigenic structure of the modified protein, in our experience, the detection of anti-DFS70 antibodies using IPP with the biotinylated recombinant protein is largely consistent with their detection by WB with bacterial recombinant protein [17]. Detections of anti-MDA-5 and anti-TIF1-γ antibodies using IPP with the biotinylated recombinant protein are also closely consistent with their detection by the standard IPP with radio-labeled cellular extract [18].

4. ELISA with Biotinylated Recombinant Protein

We applied the above recombinant protein biotinylated *in vitro* TnT system to ELISA. After cDNA inserted into a plasmid vector containing T7 promotor is purchased, it takes up to 10 days to construct an ELISA system for the measurement of autoantibodies (Figure 1). At the first attempt, biotinylated MDA5 recombinants were coated onto commercial ELISA plates to which streptavidin was covalently coupled via a spacer [5]. This procedure also enabled the recombinant protein to be purified from crude lysate. Although this measurement could have been done with a conventional optical system for ELISA, 10 μL of reaction mixture per well was necessary to get a sufficiently positive signal, at the cost of ~\$5/well. If we were able to purchase a commercial purified recombinant protein for less than \$100/μg, it would be more cost effective for building an ELISA. To save the cost, we used a microplate luminometer to increase the sensitivity, thereby reducing the amount of biotinylated recombinant protein

required for the assays [4, 6]. Anti-MDA5 antibody levels obtained using a luminometer had a good correlation with those obtained using spectrophotometer [19]. We were able to reduce the amount of reaction mixture of TnT from 10 μL/well to 1 μL/well. Even if the cost of streptavidin-coated plate and highly sensitive chemiluminescence substrate are added, the per-well cost will still be only around $1.

5. Anti-Mi-2 Antibodies Measured by Our ELISA

Anti-Mi-2 antibodies were the first to be identified as DM-specific marker autoantibodies [20]. Patients with DM carrying these antibodies often show classic DM skin lesions and favorable prognosis [1]. The target macromolecular protein is the Mi-2/NuRD complex, which is involved in multiple transcriptional regulatory processes [21]. Anti-Mi-2 autoantibodies mainly target Mi-2α/β that are around 240 kDa [22]. Previous epitope-mapping studies showed multiple antigenic regions on the polypeptides of Mi-2β [23]. Even the most antigenic fragment was reactive to less than 60% of anti-Mi-2-positive samples. ELISA kits for anti-Mi-2 are available from one company (Thermo Fisher Scientific Inc.), and these were validated by a single study [24]. Since full-length recombinant proteins of Mi-2α/β are not available in Japan, we tried to construct an ELISA for the measurement of anti-Mi-2 antibodies.

The full-length Mi-2β cDNA clone [25] was a kind gift from Drs. Kato and Takahashi at Nagoya University. The plasmid harboring this clone contains the T7 promotor and the HA-tag and V5/His-tag at the N-terminus and C-terminus, respectively. Biotinylated recombinant protein was produced from the cDNA, using the TnT T7 Quick Coupled Transcription/Translation System (Promega, Madison, USA) according to our protocol [4]. Nunc Immobilizer Streptavidin Plates (Thermo Scientific Nunc, Roskilde, Denmark) were prewashed 3 times with PBS containing 0.05% Tween 20 (T-PBS), coated with TnT product diluted with T-PBS (50 μL/well), and incubated for 1 hour at room temperature. After 3 washes with T-PBS, the wells were blocked with 200 μL of a blocking buffer of 0.5% bovine serum albumin (Wako, Osaka, Japan) in T-PBS for 1 hour. Uncoated wells were used to measure the background levels for each sample. Sample sera diluted with blocking buffer (50 μL/well) were incubated for 1 hour at room temperature, followed by incubation with antihuman IgG antibody conjugated with HRP (Dako, Glostrup, Denmark) (50 μL/well) at 1 : 30,000 dilution. After incubation for 1 hour at room temperature, the plates were washed and incubated with SuperSignal ELISA Femto Maximum Sensitivity Substrate (Thermo Scientific Pierce, Rockford, USA) (50 μL/well) as the substrate. Then, relative luminescence unit (RLU) was determined using the GloMax-Multi Detection System (Promega). Each serum sample was tested in duplicate, and the mean RLU-subtracted background was used for data analysis. The high-level anti-Mi-2 antibody-positive serum serially diluted to 1 : 5, starting from 1 : 500, was run as standard. Units correlated with the antibody titers of antibodies: 1 : 500 dilution, 625 units; 1 : 2,500 dilution, 125 units; 1 : 12,500 dilution, 25 units; 1 : 62,500 dilution, 5 units; 1 : 312,500 dilution, 1 unit.

From the serum bank of the Department of Dermatology, Nagoya University Hospital, we screened anti-Mi-2β antibodies in sera from 124 Japanese patients with DM (including 13 with juvenile DM, 39 with clinically amyopathic DM, and 19 with cancer-associated DM), in which 108 serum samples had been used in our previous study [4]. Five sera from patients with DM immunoprecipitated the biotinylated recombinant Mi-2β by IPP [4], and these had been confirmed to have anti-Mi-2 antibodies by IPP-WB by using anti-Mi-2α monoclonal antibody [4]. Twenty healthy individuals were assessed as normal controls. This study was approved by the Ethics Committee of the Nagoya University Graduate School of Medicine and conducted in accordance with the Declaration of Helsinki.

All 5 anti-Mi-2 positive sera identified as such by our previous study were also reactive to the recombinant in ELISA (Figure 2(a)). Using one positive serum with the high titer as the ELISA standard, all serum samples from DM patients and healthy individuals were investigated by ELISA, for which 0.5 μL/well of TnT reaction mixture was used. The cut-off level was set at 0.53 units, based on 5 standard deviations (SDs) above the mean value obtained from 20 healthy control sera. Two additional sera, which were not included in the previous study, were newly found to have anti-Mi-2 antibodies. No serum samples from the other 117 patients with DM or from the healthy individuals reached the cut-off level. One serum sample from a patient showed over 3 SDs above the mean value obtained from controls: 0.46. This serum showed no dose dependency for the amount of coated antigen (Figure 2(a)). Moreover, when only reticulocyte lysate-coated wells were used as the background for subtraction instead of uncoated wells being used, this serum unit fell below the 3SDs+mean (data not shown). Twenty ELISA-negative serum samples from DM patients were confirmed to be anti-Mi-2 negative by IPP-WB (data not shown). The clinical profiles of the 7 anti-Mi-2-positive patients are summarized and compared with data of a published Japanese multicenter study [10] in Table 1. Although ages at onset and sex ratios are different between the two studies, anti-Mi-2 positive DM is regarded as being classical DM and having a favorable prognosis without the life-threatening complication of malignancy or ILD [1].

6. Advantages and Disadvantages of the New ELISA

The results presented in this study demonstrated that a rapid, specific, sensitive, and quantitative anti-Mi-2-antibody immunoassay can be created by using commercially available *in vitro* TnT kits and precoated ELISA plates. Our newly established ELISA is a simple experimental method that does not require the use of radioisotopes. It is probably applicable for measuring various kinds of autoantibodies.

Standard IPP using cell extract has some limitations for accurate interpretation. Many DM-/PM-specific autoantigens show similar migration patterns on gel electrophoresis:

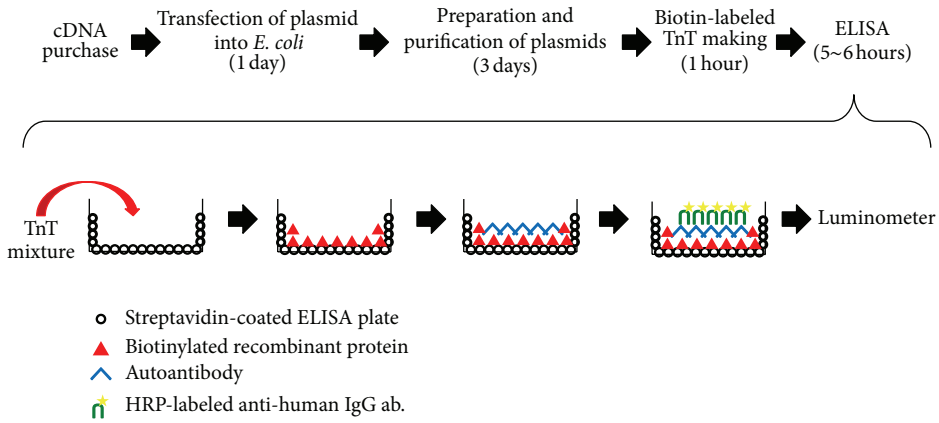

Figure 1: ELISA development using biotinylated recombinant protein. The process flow summarizes the method of ELISA construction, from obtaining the cDNA to obtaining the data by luminometer. We perform phenol/chloroform treatments twice to inhibit RNase for the plasmid purification.

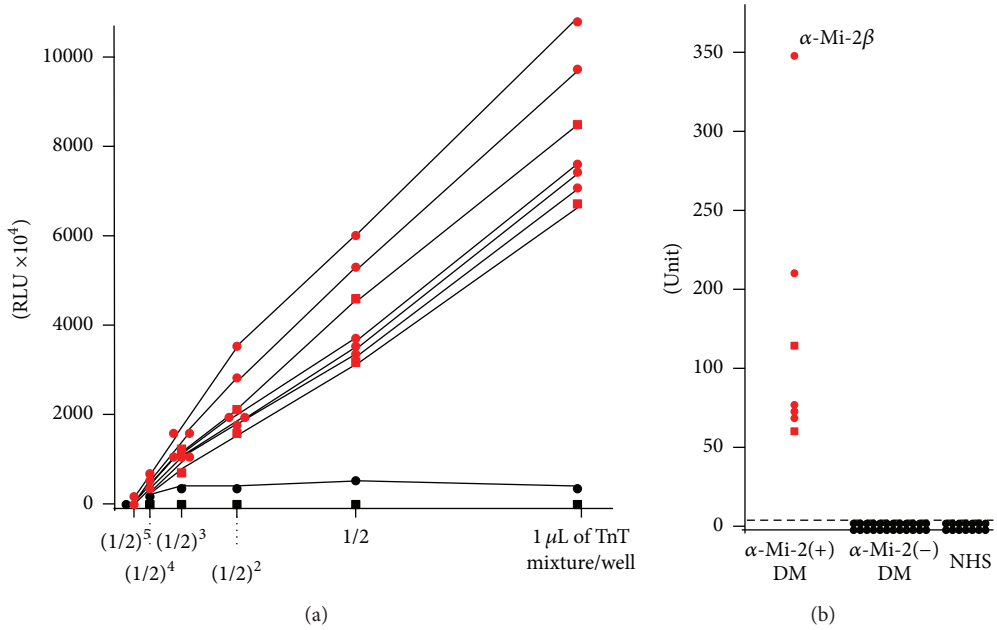

Figure 2: ELISA using biotinylated recombinant Mi-2β protein. (a) Serial dilution of biotinylated *in vitro* translation and transcription product for ELISA. Red circles: anti-Mi-2 positive sera defined in our previous analysis. Red squares: newly identified anti-Mi-2 positive sera. Black circles: serum from patients with DM having high background. Black squares: healthy individual serum. Recombinant protein was diluted with T-PBS to 50 μL of the final volume per well. Serum dilution was 1 : 1,000. RLU = relative luminescence unit. (b) Measurement of anti-Mi-2β antibodies in 128 serum samples from patients with DM or 20 healthy control subjects (NHS). We used the 0.5 μL/well of TnT mixture and patient serum samples diluted to 1 : 1000 for measuring all samples. Antibody units were calculated from the RLU using a standard curve obtained from serial concentrations of a serum sample containing a high titer of the anti-Mi-2β antibody. The broken line indicates the cut-off value (0.53 units).

from 100 kDa to 200 kDa. Some antigens may be insufficiently expressed in standard cultured cell lines. Although many commercial measuring kits can be purchased, commercial laboratory kits for new diagnostic autoantibodies are not readily available [26]. Traditional methods that use purified recombinant proteins are labor intensive and may take months to obtain proteins of sufficient purity and optimization prior to ELISA development. This process limits the rapid serologic analysis of novel antigens. Recently, many purified recombinant proteins have become commercially available. However, they are usually expensive, and the various kinds of epitope tags depend on each company. It is often difficult to find commercially available recombinant proteins of large molecule size as full-length proteins. For example, Mi-2 antigens are >200 kDa, and their full-length recombinants are not yet commercially available in Japan.

Table 1: Comparison of clinical data for patients with anti-Mi-2 antibody in a previous report and in the present study.

	Multicenter study*	This study
Anti-Mi-2 (+) pts., number (%)	9/376 (2.4)	7/124 (5.6)
Age at onset, median (range), y	45 (16–66)	62 (40–73)
Sex, M/F, number	6/3	1/6
Diagnosis, %		
Classical DM	100	100
Clinically amyopathic DM	0	0
Clinical features, %		
Muscle weakness	100	100
Arthritis	11	14
ILD	11	0
Malignancy	0	0
Skin eruptions, %		
Heliotrope rash	67	57
Facial erythema**	56	100
Gottron sign	89	100
Prognosis (alive), %	100	100

*Data from [10]. This cohort includes 7 patients used in this study, all of whom were negative for anti-Mi-2 antibodies. **Facial erythema other than heliotrope rash.

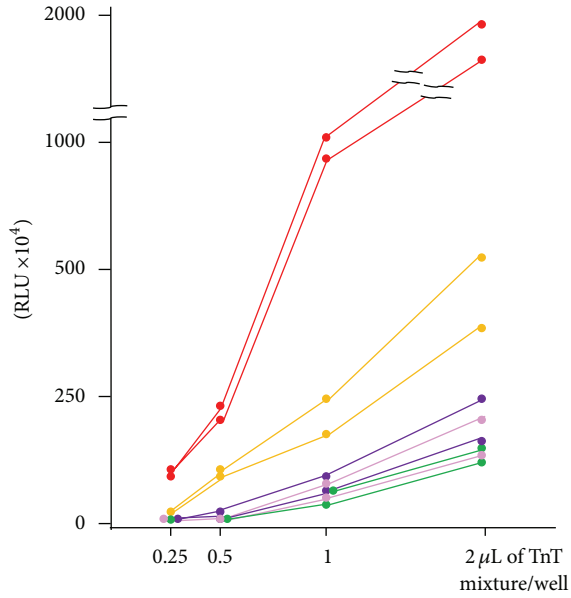

Figure 3: ELISA using biotinylated recombinant proteins of 5 different DM-specific autoantigens. Serial dilution of biotinylated *in vitro* translation and transcription product for ELISA using 2 representative positive sera from DM patients. Serum dilution was 1 : 1,000. Red circles: Mi-2β. Yellow circles: MJ (NXP-2). Purple circles: TIF1-α. Pink circles: MDA5. Green circles: TIF1-γ. RLU = relative luminescence unit.

In some countries, various recombinant DM autoantigens, including Mi-2β, have recently become commercially available, for example, from Diarect AG (Freiburg, Germany) and SurModics, Inc. (Eden Prairie, MN, USA).

Our assay has several limitations for detecting antibodies. When recombinant proteins are prepared by *in vitro* TnT, post-translational modification does not fully occur. Some autoantibodies are regarded as recognizing the post-translational modification of the protein [27]. Autoantibodies to insulinoma-associated protein 2 (IA-2), which are a serological marker of insulin-dependent diabetes mellitus, preferentially react to baculovirus-expressed IA-2 slightly better than *in vitro* translated IA-2 reacts [28, 29]. Autoantibodies to thyrotropin receptor (TSHR) in Graves' disease do not efficiently bind to the TSHR recombinant produced in an *in vitro* TnT system [30, 31]. The above data are probably due to the glycosylation of IA-2 and TSHR, which plays an important role in autoepitopes of these antigens and may occur in baculovirus expression system but not in TnT system.

The expected amounts of recombinant protein derived from the positive control plasmid are in the range of ~ 300 ng of protein in a standard 50 μL reaction, according to manufacturer's instructions. Protein expression with *in vitro* TnT can vary from batch to batch, and quantification of the resulting protein concentration is challenging. Estimating incorporation levels of biotinylated lysine is more difficult. Although large proteins are sometimes difficult to express as recombinant full product, we succeeded in producing Mi-2β by using a TnT system [4]. Recently, we have also succeeded in producing larger autoantigens, for example, envoplakin and periplakin, by using this system [32].

The biggest potential issue with our system is that the presence of biotinylated lysines may affect epitope recognition by the autoantibodies. We compared our ELISA results of representative positive sera for 5 different DM-marker autoantigens: Mi-2β, MJ (NXP2), MDA5, TIF1-α, and TIF1-γ (Figure 3). Recombinant Mi-2β was much more highly reactive than the other antigens. Although the reason is obscure, several possibilities are considered. The ratio of lysine content in Mi-2β (9.2%) is the highest among these antigens and, interestingly, the sequence has 3 short lysine stretches consisting of 5 or 6 residues at the N-terminus, which may incorporate biotin-labeling efficiently.

An improved method can be considered that uses TnT recombinants without biotinylation. Tag polypeptide and its ligand for coating tagged proteins, the binding of which is as strong as biotin-streptavidin binding, can be applied, such as Halo tag and its ligand [33]. We plan to investigate whether an ELISA constructed with a cDNA clone inserted into the Halo tag vector containing T7 promotor and with ligand-coated plate improves the reactivities of autoantibodies, including anti-TIF1-α and anti-TIF1-γ antibodies.

Conflict of Interests

The authors have no conflict of interests to declare.

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Research Article

Highlights on Novel Technologies for the Detection of Antibodies to Ro60, Ro52, and SS-B

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Objective. We aimed to compare a chemiluminescent immunoassay (CIA, QUANTA Flash) on BIO-FLASH with a multiplex flow immunoassay (MFI) on BioPlex 2200 for the detection of antibodies to Ro60, Ro52, and SS-B. *Methods*. The study included 241 samples, from patients suffering from systemic autoimmune diseases ($n = 108$) as well as disease controls ($n = 133$). All samples were tested for anti-Ro52, anti-Ro60, and anti-SS-B (La) antibodies on QUANTA Flash (INOVA Diagnostics, San Diego, USA) and BioPlex 2200 (Bio-Rad Laboratories Inc., Hercules, USA). Discrepant samples were tested by two independent methods: BlueDot/ANA and QUANTRIX Microarray (both D-tek, Belgium). *Results*. The overall qualitative agreements were 95.4% (95% confidence interval, CI 92.0–97.7%) for anti-Ro52, 98.8% (95% CI 96.4–99.7%) for anti-Ro60, and 91.7% (95% CI 87.5–94.9%) for anti-SS-B antibodies. There were 34 discrepant samples among all assays (20 anti-SS-B, 11 anti-Ro52, 3 anti-Ro60). 30/33 of retested samples (by D-tek dot blot) agreed with the QUANTA Flash results. Similar findings were obtained with QUANTRIX Microarray kit. *Conclusion*. QUANTA Flash and BioPlex 2200 show good qualitative agreement. The clinical performances were similar for anti-Ro52 and anti-Ro60 autoantibodies while differences were observed for anti-SS-B (La) antibodies.

1. Introduction

Autoantibodies targeting extractable nuclear antigens (ENA) are hallmarks in the diagnosis of systemic autoimmune rheumatic disease (SARD) such as systemic lupus erythematosus (SLE), Sjögren's syndrome (SjS), systemic sclerosis (SSc), polymyositis/dermatomyositis (PM/DM), and mixed connective tissue disease (MCTD). In addition, anti-ENA antibodies can be detected in undifferentiated connective tissue disease (UCTD) [1]. The primary antigenic targets of anti-ENA antibodies are U1-ribonucleoproteins (RNP), Sm (Smith antigen) [2], Scl-70 (topoisomerase I) [3], Jo-1, Ro60 (SS-A) [4], Ro52 (TRIM21) [4], and SS-B (La) [1]. Not all of those antibodies are specific for a particular disease but are useful to help ruling in or out SARD [1]. Among the most common autoantibodies are those to Ro52, Ro60 and SS-B [1]. Historically, anti-Ro52 and anti-Ro60 antibodies combined have been detected and reported [4]. However, recent data suggested that both the cellular function of the two proteins and the disease association of anti-Ro52 and anti-Ro60 antibodies are significantly different [1, 4, 5]. In addition, about 20% of those antibodies can be missed when tested using a blend of the two antigens [4]. Besides the diagnostic value of antibodies to Ro52, Ro60, and SS-B, it has been shown that those antibody specificities can precede the clinical onset of SLE for many years [6]. In addition, antibodies to the three antigens characterize a subpopulation of SLE patients that are clinically different from other SLE patients [7]. Several assays have been developed and used for the detection of anti-ENA antibodies including ELISA, line immunoassays (LIA) [8], multiplex flow immunoassay (MFI) mostly referred to as addressable laser bead assays (ALBIA) [9–12] and protein arrays [13]. In recent years, the chemiluminescence technology, which has been used

for clinical chemistry for more than 10 years, has been applied for autoantibody testing [14, 15]. The objective of the present study was to analyze the performance of novel chemiluminescent immunoassays (CIA, QUANTA Flash) on BIO-FLASH, a rapid-response chemiluminescent analyzer in comparison with multiplex flow immunoassay on BioPlex 2200 system for the detection of antibodies to Ro60, Ro52, and SS-B. Additionally, the clinical utility of antibody titer and multiple positivity [16] were analyzed.

2. Materials and Methods

2.1. Sera. The study included 241 samples from patients suffering from SARD ($n = 108$) as well as disease controls ($n = 133$). All samples were tested for anti-Ro52, anti-Ro60, and anti-SS-B antibodies by QUANTA Flash (INOVA Diagnostics, San Diego, USA) and BioPlex 2200 (Bio-Rad Laboratories Inc., Hercules, USA). Discrepant samples were tested by two independent methods: BlueDot/ANA and QUANTRIX Microarray (both D-tek, Belgium). The diagnoses were established as described before [17] or according to the standard disease criteria.

This study meets and is in compliance with all ethical standards in medicine, and informed consent was obtained from all patients according to the Declaration of Helsinki.

2.2. QUANTA Flash Assays. The QUANTA Flash assays (INOVA Diagnostics Inc., San Diego, CA, USA) are novel CIAs that are used on the BIO-FLASH instrument (Biokit S.A., Barcelona, Spain), fitted with a luminometer, as well as all the hardware and liquid handling accessories necessary to fully automate the assay. The principle of the BIO-FLASH system has recently been described [14, 15]. The QUANTA Flash assays for this study were developed using recombinant antigens (INOVA Diagnostics, see Table 1) coated onto paramagnetic beads. Prior to use, the lyophilized beads are resuspended using the resuspension buffer. A patient serum sample is prediluted with the BIO-FLASH sample buffer in a small disposable plastic cuvette. Small amounts of the diluted patient serum, the beads, and the assay buffer are all combined into a second cuvette, mixed, and then incubated for 9.5 minutes at 37°C. The magnetized beads are sedimented using a strong magnet in the washing station and washed several times followed by addition of isoluminol conjugated anti-human IgG and again incubated 9.5 minutes at 37°C. The magnetized beads are sedimented and washed repeatedly. The isoluminol conjugate is oxidized when sodium hydroxide solution and peroxide solutions ("Triggers") are added to the cuvette, and the flash of light produced from this reaction is measured as Relative Light Units (RLUs) by the BIO-FLASH optical system. The RLUs are proportional to the amount of isoluminol conjugate that is bound to the human IgG, which is in turn proportional to the amount of autoantibodies bound to the antigen on the beads.

2.3. BioPlex 2200. BioPlex 2200 (Bio-Rad, Hercules, CA) system is an automated analyzer that uses multiplex bead technology (Luminex, Austin, TX, US) to simultaneously detect antibodies to several antigens in a single tube. The BioPlex 2200 ANA Screen is intended for the qualitative screening of ANA, the quantitative detection of antibody to dsDNA, and the semiquantitative detection of ten separate antibodies (Chromatin, Ribosomal P, SS-A, SS-B, Sm, SmRNP, RNP, Scl-70, Jo-1, and Centromere B) [10, 11] in human serum and/or EDTA or heparinized plasma. The test system is used as an aid in the diagnosis of SARD. The system reports anti-Ro52 and anti-Ro60 antibodies as individual results outside the United States and the combined result as anti-SS-A in the United States due to lack of 510 K clearance by the Food and Drug Administration (FDA) of the anti-Ro52 antibody assay. Characteristics of the assay are summarized in Table 1.

2.4. QUANTRIX and Dot Blot. ANA12 IgG BlueDot (ANA12D+DFS70) and ANA PROFILE 25 Ag DOT (Code: AD ANA25DBD) for BlueDiver Instrument (both D-tek, Belgium) were used as comparator methods on discrepant samples. ANA12 IgG BlueDot contains the antigens: Nucleosome, Sm, RNP (68 kD/A/C), Ro60, Ro52, SSB(La), Jo-1, Scl-70, CENP-A/B, PCNA, Ribosome P(P0), and DFS70. For this study, only anti-Ro60, anti-Ro52, and anti-SS-B antibodies were used. The test procedure followed the instruction for use (see http://www.d-tek.be/). ANA PROFILE 25 Ag DOT contains the antigens: Nucleosome, dsDNA, Histones, Sm, RNP, Sm/RNP, Ro60, Ro52, SSB(La), Scl-70, Ku, PM/Scl-100, Mi-2, Jo-1, PL-7, PL-12, SRP, Ribosome P(P0), CENP-A/B, PCNA, sp100, gp210, M2 recombinant, M2/nPDC, and f-actin. For this study, only Ro60, Ro52, and SS-B were used.

2.5. Statistical Analyses. The data were statistically evaluated using the Analyse-it software (Version 1.62; Analyse-it Software, Ltd., Leeds, UK). Chi-square, Spearman's correlation, and Cohen's *kappa* agreement test were carried out to analyze the agreement between portions, and P values < 0.05 were considered significant. Receiver operating characteristics (ROC) analysis was used to analyze the discriminatory ability of different immunoassays. Cluster analysis was used to illustrate the relationship between different assays [18] and to display the reactivity pattern of the patients. Hierarchical clustering was performed using average linkage clustering where patient correlation was performed uncentered and the reactivities uncentered.

3. Results

3.1. Correlation between QUANTA Flash and BioPlex 2200. The overall qualitative agreements between QUANTA Flash and BioPlex 2200 were 95.4% (95% confidence interval, CI 92.0–97.7%) for anti-Ro52, 98.8% (95% CI 96.4–99.7%) for anti-Ro60, and 91.7% (95% CI 87.5–94.9%) for anti-SS-B antibodies (Table 2). Using ROC analyses with the BioPlex 2200 results as the comparator, excellent agreement was found for anti-Ro60, good for anti-Ro52, and moderate for anti-SS-B antibodies. Areas under the curve (AUC) values

Table 1: Sensitivities and specificities of the different assays in different diseases.

	QUANTA Flash Ro52	BioPlex 2200 Ro52	QUANTA Flash Ro60	BioPlex 2200 Ro60	QUANTA Flash SS-B	BioPlex 2200 SS-B
Antigen source	Recombinant, insect cells	Recombinant, insect cells	Recombinant, insect cells	Native	Recombinant, insect cells	Native
Analytical measuring range (cut-off)	2.3–1685.3 CU (20 CU)	0.2–8.0 units (1.0 units)	4.9–1374.8 CU (20 CU)	0.2–8.0 units (1.0 units)	3.3–1706.8 CU (20 CU)	0.2–8.0 units (1.0 units)
Sensitivity in SARD % (95% CI)	47.2 (37.5–57.1)	40.7 (31.4–50.6)	65.7 (56.0–74.6)	68.5 (58.9–77.1)	24.1 (16.4–33.3)	33.3 (24.6–43.1)
Sensitivity in SLE % (95% CI)	50.0 (28.2–71.8)	45.5 (24.4–67.8)	68.2 (45.1–86.1)	68.2 (45.1–86.1)	31.8 (13.9–54.9)	45.5 (24.4–67.8)
Sensitivity in SjS % (95% CI)	51.9 (37.8–65.7)	44.4 (30.9–58.6)	75.9 (62.4–86.5)	75.9 (62.4–86.5)	31.5 (19.5–45.6)	31.5 (19.5–45.6)
Specificity % (95% CI)	92.5 (86.6–96.3)	95.5 (90.4–98.3)	93.2 (87.5–96.9)	93.2 (87.5–96.9)	97.7 (93.5–99.5)	94.7 (89.5–97.9)

Table 2: Qualitative agreements between QUANTA Flash and BioPlex assays.

(a)

All patients (n = 241)	BioPlex 2200 Ro52			Percent agreement (95% confidence interval)
	Positive	Negative	Total	
QUANTA Flash Ro52				
Positive	50	11	61	Pos agreement = 100.0% (92.9–100.0%)
Negative	0	180	180	Neg agreement = 94.2% (89.9–97.1%)
Total	50	191	241	Total agreement = 95.4% (92.0–97.7%)
				kappa = 0.87 (95% CI 0.80–0.95)

(b)

All patients (n = 241)	BioPlex 2200 Ro60			Percent agreement (95% confidence interval)
	Positive	Negative	Total	
QUANTA Flash Ro60				
Positive	80	0	80	Pos agreement = 96.4% (89.8–99.2%)
Negative	3	158	161	Neg agreement = 100.0% (97.7–100.0%)
Total	83	158	241	Total agreement = 98.8% (96.4–99.7%)
				kappa = 0.97 (95% CI 0.94–1.00)

(c)

All patients (n = 241)	BioPlex 2200 SS-B			Percent agreement (95% confidence interval)
	Positive	Negative	Total	
QUANTA Flash SS-B				
Positive	26	3	29	Pos agreement = 60.5% (44.4–75.0%)
Negative	17	195	212	Neg agreement = 98.5% (95.6–99.7%)
Total	43	198	241	Total agreement = 91.7% (87.5–94.9%)
				kappa = 0.68 (95% CI 0.55–0.81)

were 0.99 for anti-Ro60, 1.00 for anti-Ro52, and 0.88 for anti-SS-B antibodies (see Figure 1). Additionally good quantitative agreements were observed. The Spearman rho values were: 0.95 (95% 0.94–0.96) for anti-Ro60, 0.75 (95% 0.69–0.80) for anti-Ro52, and 0.72 (95% 0.65–0.78) for anti-SS-B antibodies.

3.2. Clinical Performance of QUANTA Flash and BioPlex 2200. The prevalence and titers of anti-Ro52, anti-Ro60, and anti-SS-B antibodies in different cohorts using both assay methods can be found in Figure 2. Comparative ROC analyses were performed on all assays for patients with SARD compared with controls and showed similar results for QUANTA Flash and BioPlex 2200 for anti-Ro60 antibodies (see Figure 3). For anti-Ro52 antibodies the AUC value was significantly higher for QUANTA Flash compared to BioPlex 2200 (0.82 versus 0.69; $P < 0.0001$). However, the difference

Table 3: Overview of discrepant samples sorted according to disease state.

Sample ID	Diagnosis	QUANTRIX (cut-off = 6 U/mL)	D-tek Dot Blot	QUANTA Flash Ro52 (cut-off = 20 CU)	BioPlex 2200 (Ro52) (cut-off = 1.0)
ASX~0039	SjS	Not tested	1	30.2	0.2
ASX~0068	SjS	12	1	30.8	0.5
BEx0026	SjS	12	1	25	0.7
BEx0042	SjS	14	1	27.6	0.9
BEx0040	SLE	7	1	24.3	0.2
BEx0046	UCTD	10	1	56.4	0.4
BEx0058	UCTD	0	0	21.4	0.2
ASX~0088	RA	40	1	254.9	0.2
BSX0053	RA	Not tested	1	200.2	0.2
BSX0056	RA	Not tested	1	55.6	0.2
BSX0035	AT	Not tested	0	69.2	0.2
Sample ID	Diagnosis	QUANTRIX (cut-off = 6 U/mL)	D-tek Dot Blot	QUANTA Flash Ro60 (cut-off = 20 CU)	BioPlex 2200 (Ro60) (cut-off = 1.0)
BEx0006	UCTD	Not tested	0	4.9	8
BEx0051	UCTD	0	0	4.9	1.2
BEx0056	UCTD	0	0	8.2	1.1
Sample ID	Diagnosis	QUANTRIX (cut-off = 6 U/mL)	D-tek Dot Blot	QUANTA Flash SSB (cut-off = 20 CU)	BioPlex 2200 (SSB) (cut-off = 1.0)
BEx0001	SjS	0	0	8.4	3
BEx0014	SjS	0	0	24.8	0.9
BEx0035	SjS	0	0	13.1	8
BEx0036	SjS	Not tested	Not tested	25.5	0.2
ASX~0076	SLE	0	0	16.6	8
ASX~0082	SLE	0	0	8.5	8
BEx0054	SLE	0	0	3.3	1
BEx0013	UCTD	Not tested	0	4.2	8
BEx0022	UCTD	0	0	3.3	1.2
BEx0030	UCTD	Not tested	0	3.3	8
BEx0033	UCTD	Not tested	1	225.8	0.2
BEx0037	UCTD	0	0	5.3	1.9
BEx0058	UCTD	0	0	5.5	1.2
BEx0067	UCTD	0	0	5.1	4.7
BEx0068	UCTD	0	0	3.3	1.8
BEx0069	UCTD	0	0	3.3	2.7
ASX~0098	RA	0	0	3.3	1.2
BEx0060	RA	0	0	16	2.4
BSX0043	RA	0	0	3.3	4.2
BSX0091	HI	0	0	3.3	1.1

AT: atopic dermatitis; HI: healthy individual; UCTD: undifferentiated connective tissue disease; RA: rheumatoid arthritis; SjS: Sjögren syndrome; SLE: systemic lupus erythematosus.

was found in the nonclinically relevant area of the AUC. For anti-SS-B antibodies the AUC value was slightly higher for QUANTA Flash compared to BioPlex 2200 (0.73 versus 0.69; $P > 0.05$). The sensitivities and specificities among SARD, SLE, and SjS patients are shown in Table 1.

3.3. Analysis of Discrepant Samples. Discrepant samples (20 anti-SS-B, 11 anti-Ro52, 3 anti-Ro60) with sufficient residual volume ($n = 33$) were tested by two comparative methods. Thirty out of 33 discrepant samples among all assays (19 anti-SS-B, 11 anti-Ro52, 3 anti-Ro60) retested by D-tek dot blot agreed with the QUANTA Flash results (Table 3). Five of the 20 anti-SS-B discrepant samples were high positive on Bio-Plex (>8.0 units, above the AMR) and negative on QUANTA Flash, BlueDot, and QUANTRIX. Of the 17 BioPlex anti-SS-B positive/QUANTA Flash anti-SS-B negative samples 14

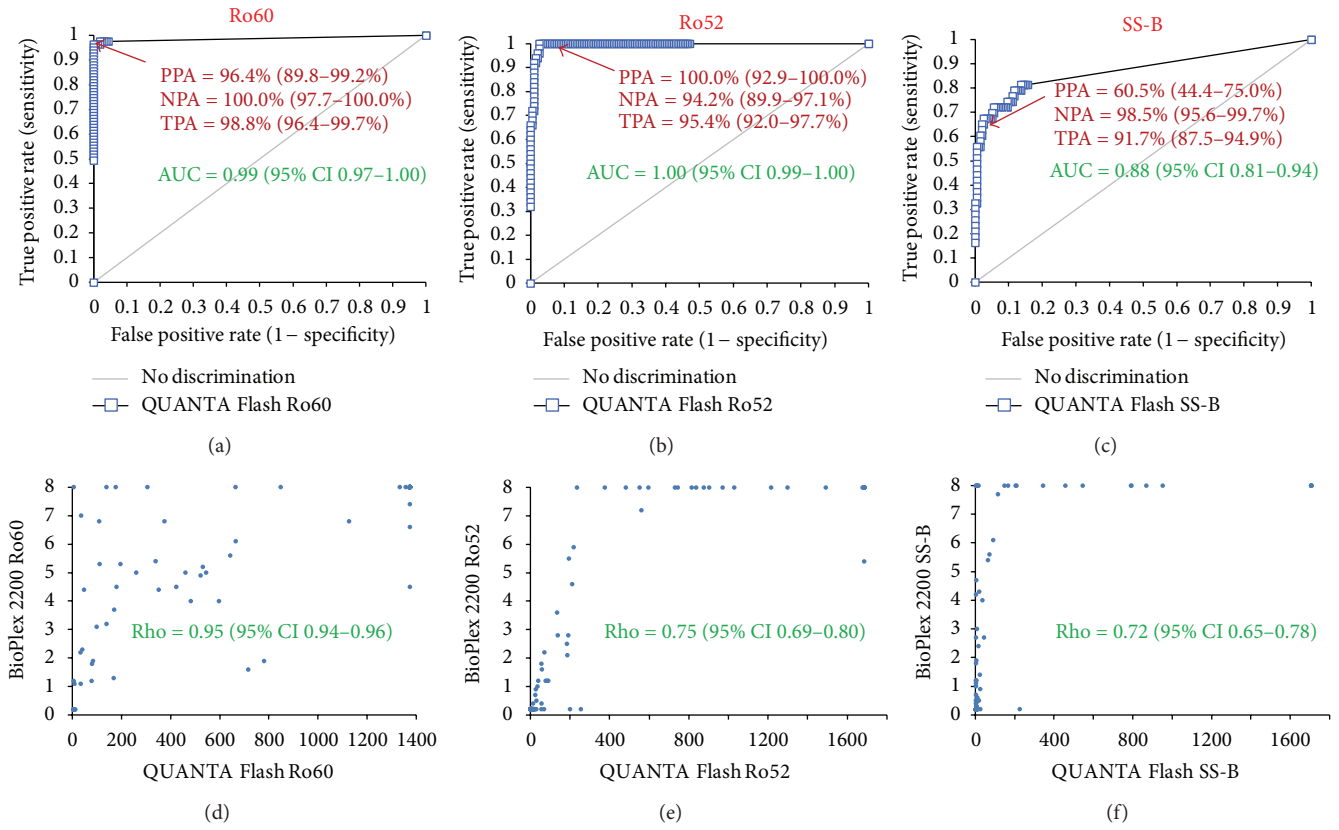


Figure 1: Comparison of QUANTA Flash with BioPlex 2200 as comparative method. Receiver operating characteristic (ROC) curves are shown in (a)–(c) and Spearman's correlation diagrams in (d)–(f). Note: A significant portion of positive samples are above the analytical measuring range of BioPlex which biases the Spearman *rho* values. PPA/NPA/TPA: Positive/Negative/Total % agreement. AUC: area under the curve.

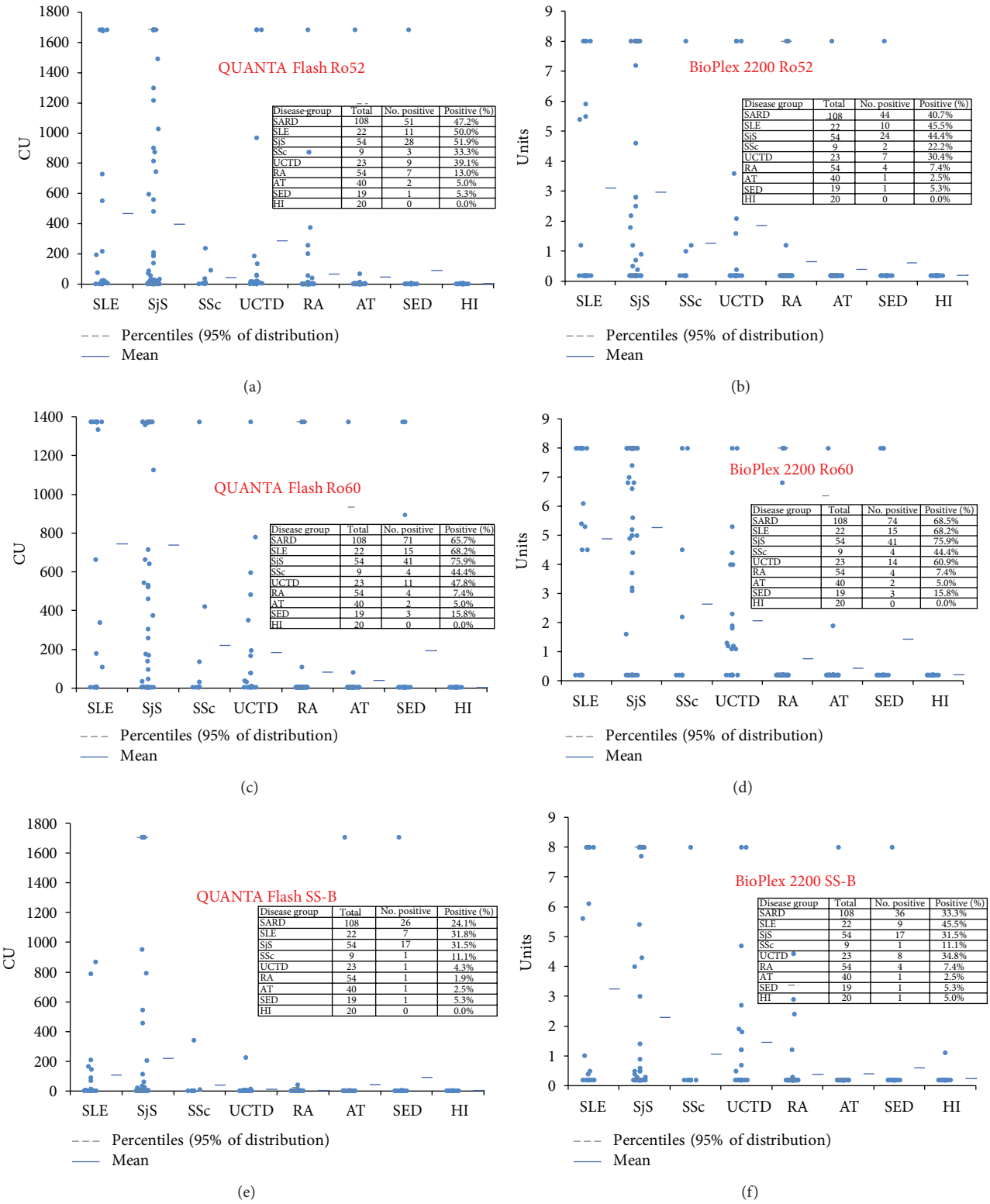
were from SARD and 4 from non-SARD patients (3 RA and one HI). All three QUANTA Flash anti-SS-B positive/BioPlex anti-SS-B negative samples were from SARD patients. All three were additionally anti-Ro60 antibody positive by both methods (QUANTA Flash and BioPlex). Figure 4 depicts a cluster analysis of all methods sorted by disease group, which serves as a visual aid of the antibody prevalence and differences between methods.

3.4. Effect of Multiple Positivity and Titer on Likelihood of Disease. To analyze the clinical utility of autoantibody titer and multiple positivity as previously described [16], we analyzed (a) the performance characteristics at the cut-off corresponding to the highest LR+ in comparison with the recommended cut-off and (b) multiple positivity (Table 4). Double positivity provided the highest LR+ for SARD compared to the controls (both for BIO-FLASH and BioPlex 2200). Highest LR+ (12.31/16.3) was obtained with SS-B at a low cut-off of 6.3 CU (QUANTA Flash)/4.3 units (BioPlex).

4. Discussion

The detection of anti-ENA antibodies is important to help in the diagnosis of SARD [1]. In recent years, several new and automated methods for the detection of anti-ENA antibodies have been developed [10, 11, 14, 15]. However, the standardization of antibody assays is still not nearly accomplished, and significant variations between different assays have been reported [19]. Since reliable detection of autoantibodies is of high importance, careful verification and validation of the assay performance is mandatory. Since the prevalence of antibodies to the other antigens contained in the BioPlex 2200 ANA profile was low and did not allow for statistical evaluation, we focused on the comparison between QUANTA Flash and BioPlex 2200 assays for the detection of anti-Ro52, anti-Ro60, and anti-SS-B antibodies.

A broad range of line immunoassays (LIA) and dot-blot assays are available and are usually used to confirm previous identified autoantibodies [20, 21]. Mainly manually performed, these assays offer a simple way of multiplex testing and even automated LIAs have become available [8]. Therefore, we choose a Dot blot assay (variation of LIA) and a microarray as confirmation assays for the discrepant samples. Multiplex assays based on the Luminex technology use addressable laser beads and are therefore often referred to as ALBIA (addressable laser bead assays) or multiplex flow immunoassay (MFI) [9]. Today several commercial MFI kits are available for the detection of autoantibodies to nuclear antigens [10, 11, 22, 23]. Similar to LIAs, the number of antigens and the antigen composition significantly varies.



Disease group	Total	No. positive	Positive (%)
SARD	108	51	47.2%
SLE	22	11	50.0%
SjS	54	28	51.9%
SSc	9	3	33.3%
UCTD	23	9	39.1%
RA	54	7	13.0%
AT	40	2	5.0%
SED	19	1	5.3%
HI	20	0	0.0%

Disease group	Total	No. positive	Positive (%)
SARD	108	44	40.7%
SLE	22	10	45.5%
SjS	54	24	44.4%
SSc	9	2	22.2%
UCTD	23	7	30.4%
RA	54	4	7.4%
AT	40	1	2.5%
SED	19	1	5.3%
HI	20	0	0.0%

Disease group	Total	No. positive	Positive (%)
SARD	108	71	65.7%
SLE	22	15	68.2%
SjS	54	41	75.9%
SSc	9	4	44.4%
UCTD	23	11	47.8%
RA	54	4	7.4%
AT	40	2	5.0%
SED	19	3	15.8%
HI	20	0	0.0%

Disease group	Total	No. positive	Positive (%)
SARD	108	74	68.5%
SLE	22	15	68.2%
SjS	54	41	75.9%
SSc	9	4	44.4%
UCTD	23	14	60.9%
RA	54	4	7.4%
AT	40	2	5.0%
SED	19	3	15.8%
HI	20	0	0.0%

Disease group	Total	No. positive	Positive (%)
SARD	108	26	24.1%
SLE	22	7	31.8%
SjS	54	17	31.5%
SSc	9	1	11.1%
UCTD	23	1	4.3%
RA	54	1	1.9%
AT	40	1	2.5%
SED	19	1	5.3%
HI	20	0	0.0%

Disease group	Total	No. positive	Positive (%)
SARD	108	36	33.3%
SLE	22	9	45.5%
SjS	54	17	31.5%
SSc	9	1	11.1%
UCTD	23	8	34.8%
RA	54	4	7.4%
AT	40	1	2.5%
SED	19	1	5.3%
HI	20	1	5.0%

FIGURE 2: Prevalence and titers of anti-Ro60, Ro52, and SS-B antibodies. Results for anti-Ro60 are shown in (a) and (b), for anti-Ro52 in (c) and (d), and for anti-SS-B antibodies in (e) and (f). AT: Atopic dermatitis, HI: healthy individuals, RA: rheumatoid arthritis, SED: suspected eye disease, SLE: systemic lupus erythematosus, SjS: Sjögren's syndrome, SSc: systemic sclerosis, UCTD: undifferentiated connective tissue disease.

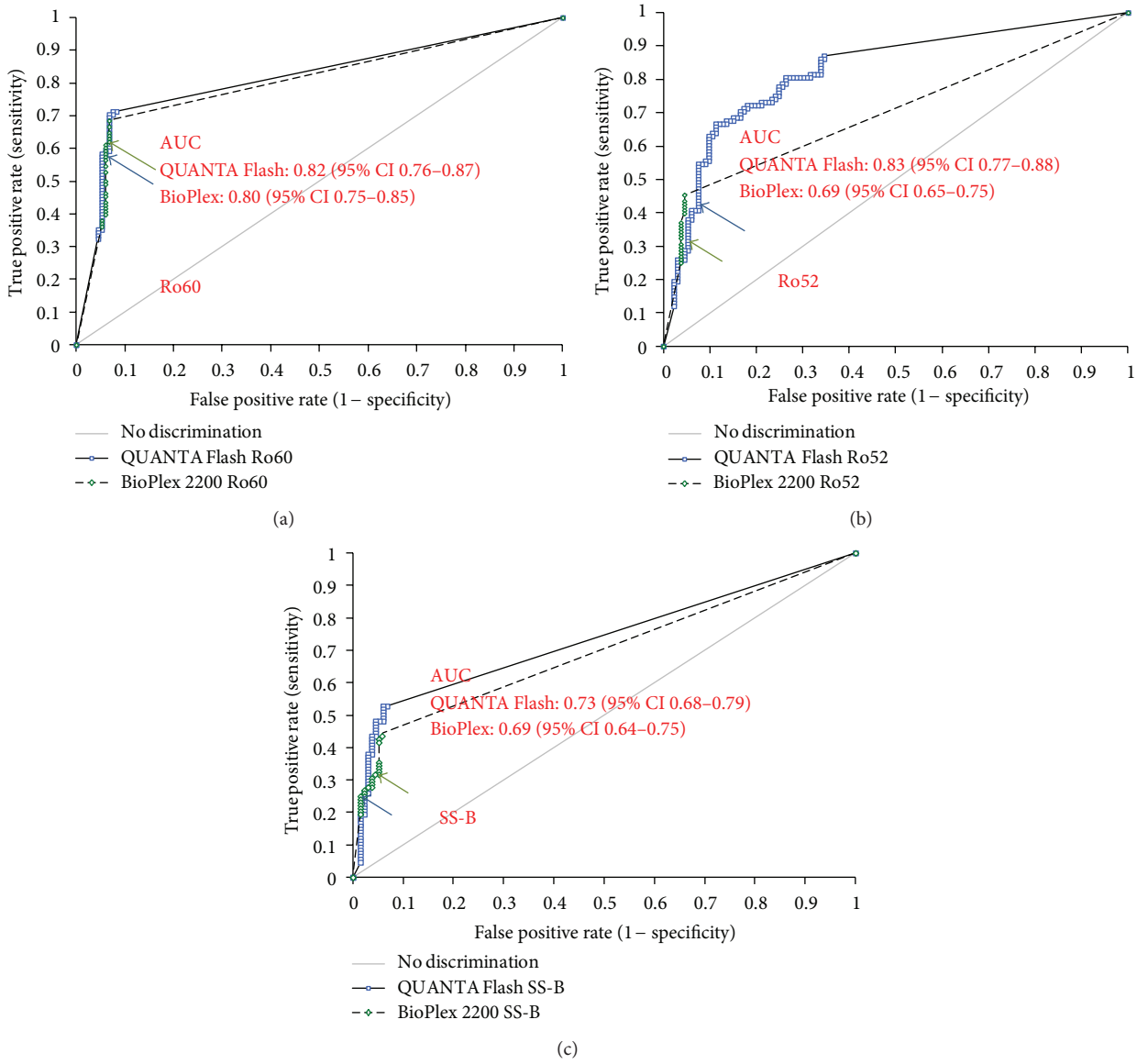


Figure 3: Clinical comparative ROC analysis. Results of patients with systemic autoimmune rheumatic diseases were compared with controls. Cut-off values are indicated by arrows. For sensitivity and specificity, see Table 1. Results for anti-Ro60 (a), anti-Ro52 (b), and anti-SS-B antibodies (c) are shown.

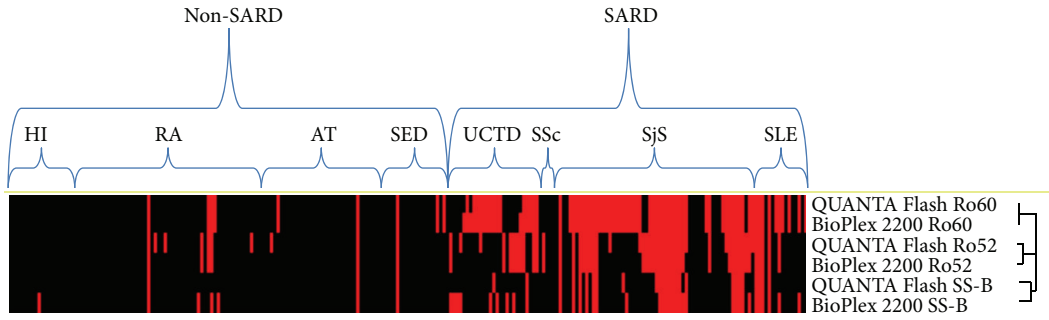


Figure 4: Supervised cluster analysis of the results. Supervised centered cluster analysis according to disease cohort is shown. The dendrogram shows that the Ro60 assays are closely related, the Ro52 assays are somewhat related, and the SS-B assays show significant difference. AT: atopic dermatitis; UCTD: undifferentiated connective tissue disease, RA: rheumatoid arthritis, SSc: systemic sclerosis; SED: suspected eye disease, SLE: systemic lupus erythematosus; HI: healthy individuals; SARD: systemic autoimmune rheumatic disease.

Table 4: Multiple positivity for Ro60, Ro52, and SS-B.

BIO-FLASH	Single positive	Double positive	Triple positive	Ro60 > 110 CU	SS-B > 6.3 CU	SS-B > 482 CU
Sensitivity	75.9%	39.8%	21.3%	58.3%	37.0%	25.0%
Specificity	89.5%	96.2%	97.7%	94.7%	97.0%	97.0%
Likelihood ratio (+)	7.21	10.59	9.44	11.08	12.31	8.31
Likelihood ratio (−)	0.27	0.63	0.81	0.44	0.65	0.77
BioPlex 2200	Single positive	Double positive	Triple positive	Ro60 > 2.2 units	SS-B > 4.3 units	SS-B > 0.4 units
Sensitivity	80.6%	38.9%	21.3%	27.8%	25.0%	45.4%
Specificity	90.2%	96.2%	97.0%	96.2%	98.5%	95.5%
Likelihood ratio (+)	8.24	10.34	7.08	7.39	16.63	10.06
Likelihood ratio (−)	0.22	0.63	0.81	0.75	0.76	0.57

First generation MFI showed polyreactivity which was caused by unspecific binding to the beads. Second generation showed significant reduced polyreactivity and thus higher specificity [24]. Multiplex assays are commonly used as a screening test for ANA or other autoantibodies. Additional methods have been developed for automated ANA detection [25–27]. Several publications have described protein arrays for the detection of autoantibodies to nuclear antigens [13]. However, until today those assays are not widely used in routine laboratories. The microarray used in this study showed good agreement with other methods and might represent a promising multiplex platform for autoantibody detection.

Although the QUANTA Flash and BioPlex 2200 use different sources of antigens (recombinant versus native for Ro60), the results are very similar. During the last decade, significant improvements have been made in recombinant protein technology [28]. In particular, novel strategies for the generation of recombinant Ro60 led to the availability of this antigen as a high quality recombinant protein [4]. Recombinant antigen manufacturing is more consistent and less dependent on the biological variations of the source material [28]. Therefore, the novel CIA shows similar assay performance combined with high degree of precision and consistency [15]. Despite the fact that both systems use recombinant Ro52 antigen, differences in the results were observed. Therefore, the difference might be related to the different antigen immobilization (bead chemistry) between the QUANTA Flash and the BioPlex 2200 system. However, our total percent agreement (95.4%, Table 2) is significantly better than agreements previously reported [5]. One putative reason for the significant difference between the QUANTA Flash and BioPlex 2200 results for anti-SS-B antibodies is the antigen source. The QUANTA Flash SS-B assay utilizes recombinant SS-B expressed in insect cells, whereas the BioPlex 2200 is based on native SS-B antigen [15]. Retesting of the discrepant samples using two additional methods mostly confirmed the QUANTA Flash results.

It is also important to point out that 5/17 BioPlex 2200 anti-SS-B positive samples (range 1–1.2 units) and 2/3 anti-SS-B positive samples by QUANTA Flash (range 20–25 CU) were low positive. Therefore, it is possible that a modified cut-off value would increase the agreement between BioPlex 2200 and QUANTA Flash SS-B. In the case of anti-SS-B discrepant samples, 16/20 are from SARD and 4 from "non-SARD" patients (3 RA and 1 HI). It is relevant that 3/4 "non-SARD" but only 3/16 of the SARD patients are only anti-SS-B positive. Since anti-SS-B antibodies occur in different SARD and no "gold standard" is available for the detection of anti-SS-B antibodies, it remains speculative which results are clinically correct and meaningful.

When compared to previous studies which analyzed the performance of the BioPlex 2200 system [10, 11], our data were in general agreement with the published data. In a large study on 510 healthy individuals, 0.2% (anti-Ro52), 0.6% (anti-Ro60), and 0.8% (anti-SS-B) were positive [10]. Despite the small number of HI used in our study, our data confirmed the specificity against HI. In a second study, the prevalence of antibodies to Ro52, Ro60, and SS-B was described [16]. Overall, the results were in agreement or not significant due to small patient groups. The QUANTA Flash assays have just been launched, and therefore only one paper has been published [16].

Historically autoantibody test results were mostly considered individually. Recently, several studies reported increased utility by combining autoantibody assay results and by considering antibody titers [16]. Consequently, we strived to analyze the synergy effect of combining the results of antibody testing for anti-Ro52, anti-Ro60, and anti-SS-B antibodies. Additionally, we analyze the impact of the antibody titers on the likelihood of disease. Although we found increased performance characteristics when combining results and with optimized cut-off values, the incremental value was not as pronounced as previously reported [16]. This might be explained by the strong overlap between the three antibodies we studied.

5. Conclusion

QUANTA Flash and BioPlex 2200 show good qualitative agreement. The clinical performances were similar for anti-Ro52 and Ro60 autoantibodies while differences were observed for anti-SSB (La) antibodies.

Highlights

(i) Good agreements between QUANTA Flash and Bio-Plex 2200 were found for anti-Ro52 and anti-Ro60.

(ii) Retesting of discrepant samples confirm in the majority of cases the QUANTA Flash results.

(iii) Antibody titer and multiple positivity might provide additional value for anti-ENA testing.

Abbreviations

AT: Atopic dermatitis
ALBIA: Addressable laser bead assays
AMR: Analytical measuring range
CIA: Chemiluminescent immunoassay
CLSI: Clinical and laboratory standards institute
CTD: Connective tissue disease
LIA: Line immunoassays
MFI: Multiplex flow immunoassay
PM/DM: Polymyositis/dermatomyositis
RA: Rheumatoid arthritis
ROC: Receiver-operating characteristics
SARD: Systemic autoimmune rheumatic disease
SED: Suspected eye disease
SLE: Systemic lupus erythematosus
SjS: Sjögren's syndrome
SSc: Systemic sclerosis
UCTD: Undifferentiated connective tissue disease.

Conflict of Interests

M. Mahler, C. Bentow, and A. Seaman are employed at INOVA diagnostics selling autoantibody assays. M. Manfredi and M. Infantino receive congress sponsor from INOVA and BIORAD. The other authors have no conflict of interests.

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