# Autoantibodies to the Rpp25 Component of the Th/To Complex are the Most Common Antibodies in Patients with Systemic Sclerosis without Antibodies Detectable by Widely Available Commercial Tests

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***ABSTRACT.*** ***Objective.*** Antinuclear antibodies (ANA) occur in up to 95% of patients with systemic sclerosis (SSc). In most, SSc-associated antibodies are detected (i.e., centromere, topoisomerase I, RNA polymerase III, PM/Scl, Ro52/TRIM21, and U1RNP). Ribonuclease P protein subunit p25, (Rpp25) is an autoantigenic component of the Th/To complex. The contribution of anti-Th/To and anti-Rpp25 antibodies to ANA positivity in patients with SSc remains unknown.

***Methods.*** Sera from 873 patients with SSc were tested for ANA, and SSc-associated antibodies were measured. Samples without antibodies to extractable nuclear antigens (ENA; n = 53, ANA+/ENA–), were analyzed by immunoprecipitation (IP) and metabolically labeled proteins and for anti-Rpp25 antibodies (n = 50) by a chemiluminescent immunoassay (CLIA) and Rpp25 ELISA.

***Results.*** Anti-Th/To antibodies occurred in 19/53 (36%), as determined by IP, and were the most common autoantibody in ANA+/ENA– SSc. Of those samples, 50/53 were available for additional testing by CLIA and ELISA. Anti-Rpp25 antibodies were detected in 12 (24% CLIA) or 10 (20% ELISA) of 50 patients. Receiver-operating characteristic curve analysis showed similar discrimination between Th/To IP-positive (n = 19) and -negative samples (n = 31) by CLIA and ELISA (area under the curve 0.90 vs 0.87; p = 0.6691). The positive percent agreement between IP and CLIA or ELISA was 12/19 (63.2%, 95% CI 38.4–83.7%) or 10/19 (52.6%, 95% CI 73.3–94.2%), respectively. Negative percent agreement was 100% for both assays.

***Conclusion.*** Autoantibodies to the Th/To autoantigen are important in patients with SSc who have been considered negative for SSc-specific or SSc-associated antibodies by widely available commercial assays. Rpp25 can be considered a major target of anti-Th/To antibodies. Assays detecting anti-Th/To and anti-Rpp25 antibodies may be important in SSc. (First Release June 15 2014; J Rheumatol 2014;41:1334–43; doi:10.3899/jrheum.131450)

*Key Indexing Terms:*

TH/TO INDIRECT IMMUNOFLUORESCENCE SYSTEMIC SCLEROSIS
ELISA AUTOANTIBODIES

Systemic autoimmune rheumatic diseases (SARD), including systemic sclerosis (SSc), are characterized by the presence of circulating autoantibodies to intracellular antigens[1,2]. In SSc, those autoantibodies with high disease specificity include antitopoisomerase I (topo I, Scl-70)[3], anticentromere (CENP)[4], and anti-RNA polymerase III (RNAP). They represent the most important autoantibodies that are also part of the recently revised classification criteria[1,5]. Besides these, several other autoantibodies have been described including autoantibodies targeting the

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PM/Scl complex (also known as the exosome)[6], U3RNP/fibrillarin[7,8], and the Th/To autoantigens[9,10,11,12]. Anti-Th/To antibodies are one of the specificities that reportedly show homogeneous nucleolar staining in conventional indirect immunofluorescence (IIF) antinuclear antibody (ANA) tests[9,13,14]. In SSc, anti-Th/To antibodies have been associated with the limited cutaneous SSc (lcSSc) subset and the reported prevalence ranged from 1 to 13%[9,15,16]. In addition to SSc, a few reports have described anti-Th/To antibodies in rheumatoid arthritis and interstitial lung disease (ILD)[17,18].

The Th/To antigen complex is a multiprotein-RNA complex (human RNase MRP complex) consisting of a catalytic RNA and at least 10 protein components[2,10]. RNase MRP is a ubiquitously expressed eukaryotic endoribonuclease that specifically cleaves various RNA, including ribosomal, messenger, and mitochondrial RNA[10]. Almost all protein components of the RNase MRP and the evolutionarily related RNase P complex have been reported as autoantibody targets in patients with SARD[10,11,17]. Rpp25 (Ribonuclease P protein subunit p25, NP_060263.2) is a 25-kDa protein subunit of RNase P[19]. Historically, anti-Th/To antibodies have been detected by immunoprecipitation (IP)[9]. While some studies tested serological cohorts, other investigations analyzed samples initially identified based on a nucleolar IIF staining pattern. Commercial line immunoassays (LIA) for the detection of anti-Th/To antibodies based on the hPop1 target have become available and were evaluated in 2 independent studies[20,21]. In addition, an IP real-time PCR assay has been developed and evaluated[22].

Although known for over 20 years, the reported clinical association of autoantibodies to Th/To antigen components is inconsistent except for their association with lcSSc. Further, anti-Th/To antibodies are rarely used in routine testing algorithms to aid in the diagnosis and management of patients with SSc because of the unavailability of the IP assay or alternative methods. ANA play an important role in the diagnosis of SSc, being present in more than 90% of the patients. In the majority of ANA-positive patients with SSc, a spectrum of SSc-specific and SSc-associated antibodies can be detected (i.e., antibodies to CENP, topo I, RNAP, PM/Scl, Ro52/TRIM21, and U1RNP). However, in a significant portion of ANA-positive patients with SSc, no fine specificities could be detected when conventional diagnostic protocols were used[16,23]. Recently, it was found that Rpp25 was an important autoantigenic component of the Th/To complex[19,24], but the magnitude by which anti-Th/To and anti-Rpp25 antibodies contribute to ANA positivity in patients with SSc is unreported. Consequently, our present study aimed to define the prevalence of autoantibodies to Th/To and Rpp25 in SSc patients without other SSc-specific or SSc-associated antibodies.

## MATERIAL AND METHODS

*Sera*. The study subjects consisted of those enrolled in the Canadian Scleroderma Research Group (CSRG) registry, a multicenter cohort study. The subjects must have a diagnosis of SSc confirmed by a rheumatologist, be > 18 years of age, be fluent in English or French, and likely to be compliant with study procedures and visits. About 87% of subjects enrolled in the CSRG registry fulfill the 1980 American College of Rheumatology preliminary criteria for SSc, which are known to be poorly sensitive, in particular to subjects with lcSSc. The subjects included were those whose baseline visit was between September 2004 and August 2009. Ethics committee approval for the CSRG data collection protocol was obtained at McGill University (Montréal, Canada) and at all participating study sites. All subjects provided informed written consent to participate in the data collection protocol. Sera at each center were collected at the baseline registry visit, processed, and shipped to the Mitogen Advanced Diagnostics Laboratory, where they were catalogued and stored at –80°C, according to a standard operating procedure.

Sera from 873 Canadian patients with SSc were previously tested for ANA and various SSc-specific and SSc-associated antibodies including antibodies to common extractable nuclear antigens (ENA) and to those contained in an SSc line immunoassay (Euroimmun)[23,25,26]. A total of 855 samples were tested for antifibrillarin and anti-NOR90 antibodies, and 3/855 (0.35%) and 25/855 (2.9%) were positive, respectively. Samples without those antibodies (n = 53, later referred to as ANA+/ENA–) were analyzed by IP analysis of proteins and RNA and for anti-Rpp25 antibodies [n = 50 by a chemiluminescent immunoassay (CLIA), QUANTA Flash, INOVA Diagnostics] and anti-Rpp25 ELISA (University of Florida).

*Measurement of autoantibodies*. ANA were detected by IIF performed on HEp-2 substrate (HEp-2000; ImmunoConcepts) that included fluorescein-conjugated goat antibodies to human IgG (H+L). IIF patterns were detected at serum screening dilutions of 1:160 and 1:640 on a Zeiss Axioskop 2 plus (Carl Zeiss) fitted with a 100-watt USHIO super-high-pressure mercury lamp (Ushio) by 2 experienced technologists with more than 7 years of experience. Antibodies to topo I, chromatin, Sm, U1-RNP, ribosomal P, Jo-1, SSA/Ro60, and SSB-La were assayed by an addressable laser bead immunoassay using commercially available kits (QUANTA Plex ENA 8, INOVA Diagnostics Inc.; FIDIS Connective 13, TheraDiag) in a Luminex 200 (Luminex Corp.) according to the manufacturer's protocols. Antibodies to RNAP-III were detected by ELISA (QUANTA Lite RNA Pol III, INOVA Diagnostics) as were antibodies to PM/Scl (PM1 α: Dr. Fooke Laboratorien GmbH)[6]. These autoantibodies were detected by a LIA (EUROLINE, Euroimmun)[21]: CENP-A, CENP-B, fibrillarin, NOR-90, Th/To (hPop1; all sera tested were negative for Th/To by this LIA), PM/Scl-75, PM/Scl-100, Ku, platelet-derived growth factor receptor, and Ro52/TRIM21. Sera that were negative on these commercially available immunoassays were tested by IP at the University of Florida. Anti-Ki/SL antibodies were tested by ELISA using a recombinant protein PSME3 (ATGen).

Detection of anti-Th/To antibodies was based on IP confirmation of the 7-2 and 8-2 RNA by RNA analysis with urea-polyacrylamide gel electrophoresis and silver staining (Silver stain plus, Bio-Rad). Specificities were verified using previously characterized reference sera. Analysis of proteins to determine other SSc autoantibodies recognized by sera was performed by IP of $^{35}$S-methionine radiolabeled K562 cell extract, sodium dodecyl sulfate-polyacrylamide gel electrophoresis, and autoradiography as described[9,19].

*Recombinant Rpp25 antigen and anti-Rpp25 immunoassays*. Recombinant full-length, his-tagged Rpp25 was generated and purified as described and used for ELISA and CLIA[19]. For ELISA, Nunc Immobilizer Amino plates (Thermo Fisher Scientific) were coated with Rpp25 antigen and blocked with bovine serum albumin. Wells were then incubated with sera diluted 1:500 in blocking buffer for 1 h at 22°C. After being washed 3 times, wells were incubated with alkaline phosphatase conjugated donkey IgG F(ab)'2 anti-human IgG (γ-chain specific, Jackson ImmunoResearch Laboratories

Inc.). After 3 washes, plates were developed and the OD405 of each sample was converted into units based on the standard curve.

The QUANTA Flash Rpp25 (INOVA Diagnostics Inc.) assay is a novel CLIA that is currently used for research purposes only and uses the BIO-FLASH instrument (Biokit s.a.), fitted with a luminometer, as well as all the hardware and liquid-handling accessories necessary to fully automate the assay. The QUANTA Flash assay for our study was developed using full-length, purified, recombinant human Rpp25 antigen coated onto paramagnetic beads. The principle of the QUANTA Flash Rpp25 assay performed on the BIO-FLASH instrument has recently been described[19,27].

*Statistical evaluation*. The data were statistically evaluated using the Analyse-it software (Version 1.62; Analyse-it Software Ltd.). Chi-square, Spearman's correlation, and Cohen's κ 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. Descriptive statistics were used to summarize the baseline characteristics of the patients. Chi-squared tests, Fisher's exact tests, and Mann-Whitney U tests were used as appropriate. P values < 0.05 were considered statistically significant. These statistical analyses were performed with SAS v.9.2 (SAS Institute). Clustering illustrates the relationship between different assays, as described by Eisen, *et al*[28]. Hierarchical clustering was performed using the following criteria: average linkage clustering, patient correlation uncentered, and reactivities centered.

## RESULTS

*Autoantibodies detected in ANA-positive/ENA-negative sera by IP*. The majority of the ANA+/ENA– SSc patients (32/53, 60.4%) had a nucleolar staining pattern with titers ranging from 1:160 to 1:5120. Anti-Th/To antibodies were the most common antibody in ANA+/ENA– SSc patients; being found in 19/53 (36%) of the patients as determined by IP (Figure 1). All 19 anti-Th/To–positive samples had a nucleolar staining pattern and the antinucleolar titer was correlated with anti-Rpp25 antibody levels measured by ELISA (ρ = 0.49, p = 0.0003) and by CLIA (ρ = 0.48, p = 0.0005). Thus, among 32 ANA-positive samples with nucleolar staining pattern, 19 (59%) had anti-Th/To and 4 (13%) had anti-U3RNP. In addition to anti-Th/To antibodies, antibodies to topo-I were detected in 2, to U1-RNP in 2, to U3-RNP in 4, to RNA Pol I/III in 1, to NOR90 in 1, and to Su/Ago2 in 1 patient by IP. Two patients' sera that immunoprecipitated a 32kD protein and that were positive in anti-Ki/SL/PSME3 ELISA were considered anti-Ki/SL–positive.

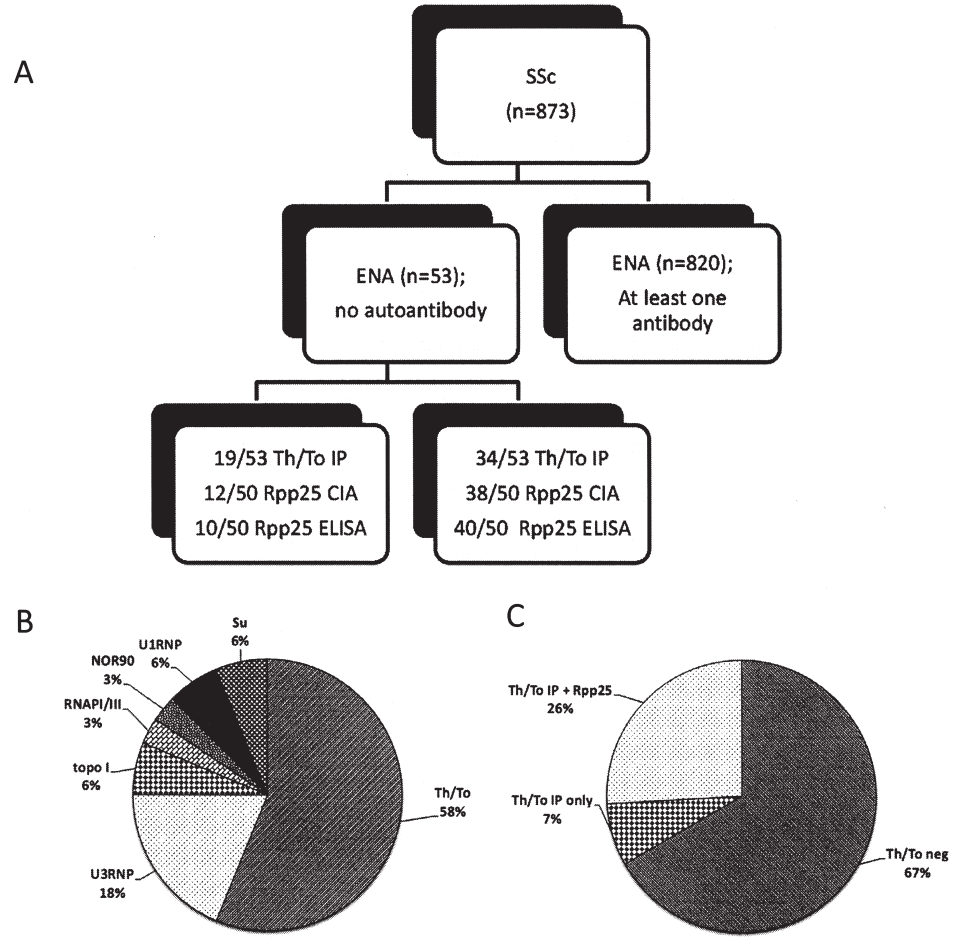


*Figure 1*. Autoantibodies in antinuclear antibody (ANA)-positive systemic sclerosis (SSc) patients without identified subserology. A. Study design and the reactivity pattern of the samples included (solid fill). A total of 53 ANA+/extractable nuclear antigens (ENA)-negative samples were tested by immunoprecipitation (IP). The autoantibody profile is shown in panel C. In panel B the reactivity to Th/To and to Rpp25 is presented. CSRG: Canadian Scleroderma Research Group; IIF: indirect immunofluorescence; CIA: chemiluminescence assay.

*Anti-Rpp25 antibodies measured by chemiluminescent technology*. A total of 50 of the 53 SSc samples were available for additional testing for anti-Rpp25 antibodies by CLIA and ELISA. Anti-Rpp25 antibodies were detected in 12 (24.0%, CLIA) or 10 (20.0%, ELISA) of 50 patients when using the recently established cutoff values[19]. When the cutoff of the ELISA was lowered, all 12 CLIA-positive samples were positive without sacrificing specificity. ROC analyses (Figure 2) showed similar discrimination between Th/To IP-positive (n = 19) and -negative samples (n = 31) by CLIA and ELISA (area under the curve 0.90 vs 0.87; p = 0.6691). The positive percent agreements between IP and CLIA or ELISA were 12/19 (63.2%) or 10/19 (52.6%), respectively. Negative percent agreements were 100% for both assays. The agreements between ELISA and CLIA were excellent.

*Cluster analysis*. Anti-Rpp25 antibodies measured by both CLIA and ELISA clustered with anti-Th/To antibodies

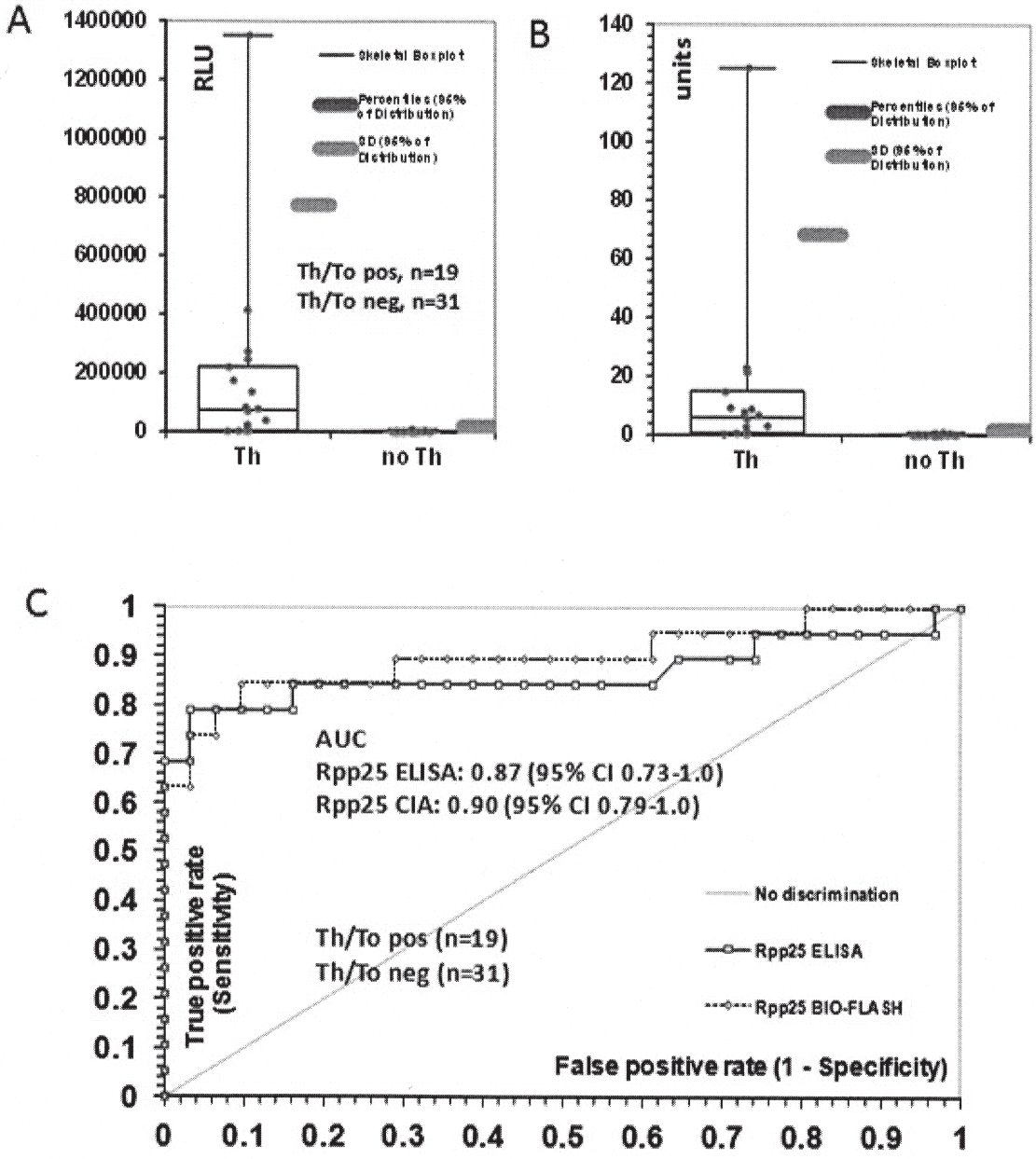


*Figure 2*. Discrimination between anti-Th/To IP-positive and -negative samples using Rpp25 ELISA and chemiluminescence assay (CIA). Comparative descriptive analyses including median values and interquartile are shown in panel A for ELISA and in panel B for CIA. Comparative receiver-operating characteristics analysis (panel C) for Rpp25 ELISA and CIA shows similar discrimination between anti-Th/To IP-positive (n = 19) and -negative patients (n = 31). IP: immunoprecipitation.

detected by IP. Almost all anti-Th/To (and anti-Rpp25) antibodies were monospecific; only 1 had anti-RNA Pol I/III antibodies in addition. Of note, anti-topo I and anti-U1-RNP antibodies clustered together, but all other antibodies exhibited significant distance (Figure 3).

*Serological-clinical associations of anti-Th/To and anti-Rpp25 antibodies in ANA-positive/ENA-negative sera*. Anti-Th/To antibodies detected by IP were negatively associated with history of myositis (0.0% vs 22.6%, p = 0.0377). Anti-Rpp25 antibodies detected by ELISA were associated with nailfold capillary change (NCC; 100.0% vs 63.2%, p = 0.0235) and interstitial lung disease (ILD; 50.0% vs 17.5%, p = 0.0460; Figure 4). Using CLIA, anti-Rpp25 antibodies showed association with NCC (100.0% vs 63.2%, p = 0.0101). When antibody titers were correlated with clinical manifestations, anti-Rpp25 antibodies determined by CLIA were associated with NCC (p = 0.0001) and ILD (p = 0.0218). In addition, negative associations with CRP levels were observed. Associations are summarized in Table 1 and Table 2.

*Serological-clinical associations of anti-Rpp25 antibodies in anti-Th/To positive sera*. Among patients with anti-Th/To antibodies (n = 19), anti-Rpp25–positive patients had a higher prevalence of ILD (50% vs 11% for ELISA, p = 0.1409; 42% vs 14% for CLIA, p = 0.3331), but the differences were not significant. In addition, anti-Rpp25–positive patients had a higher incidence of NCC (100% vs 67% for ELISA, p = 0.0867; 100% vs 57% for CLIA, p = 0.0361), suggesting that autoantibody reactivity with

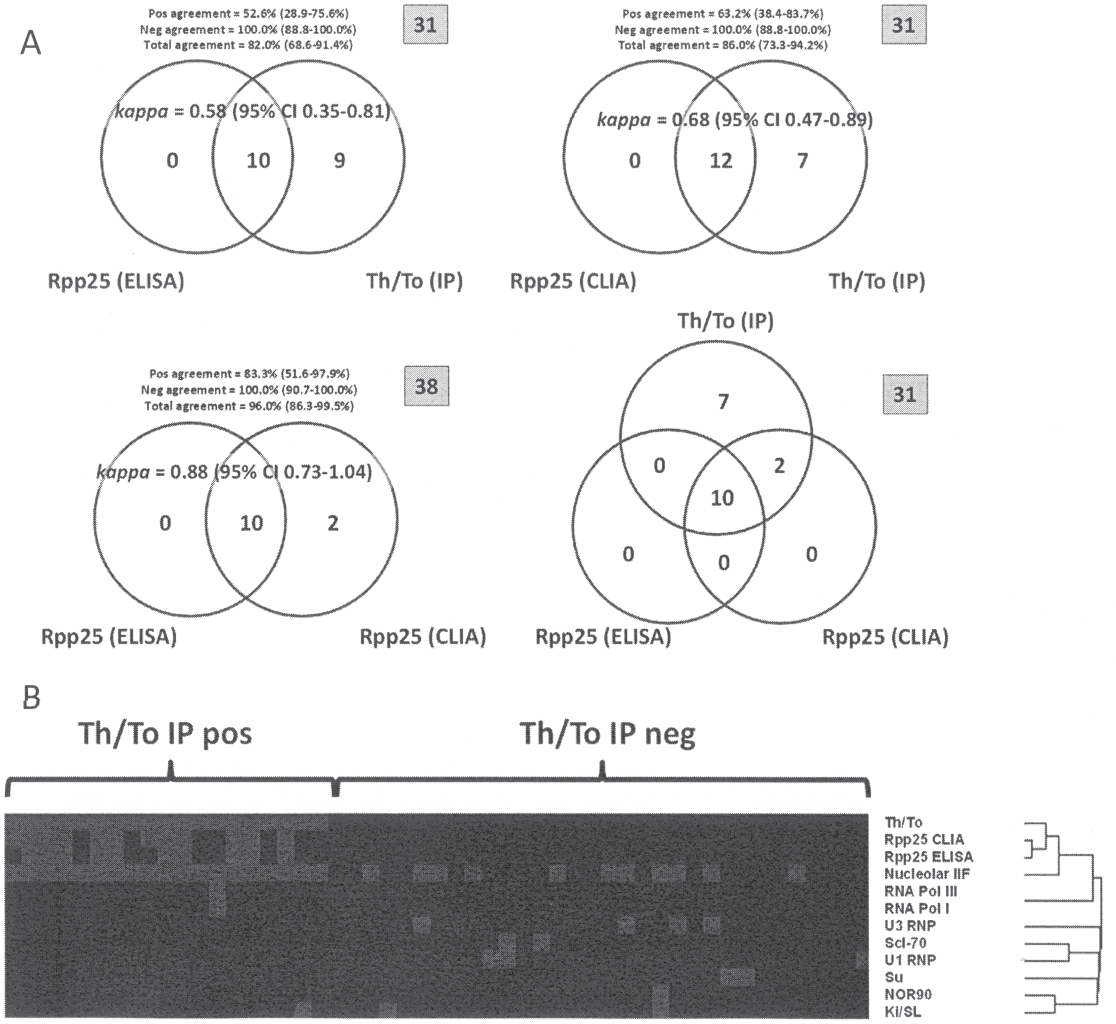


*Figure 3*. Venn diagrams and supervised cluster analysis. A. The Venn diagrams show the overlap of antibodies to Th/To detected by immunoprecipitation (IP) and anti-Rpp25 antibodies measured by ELISA and chemiluminescence assay (CLIA). B. A supervised cluster analysis shows the autoantibody profile of the antinuclear antibody–positive/extractable nuclear antigens-negative samples in a heat-map and their relation in a dendogram. Both anti-Rpp25 ELISA and CLIA cluster with the anti-Th/To results. No close relationship was found for the other autoantibodies, indicating that they are independent from anti-Th/To antibodies.

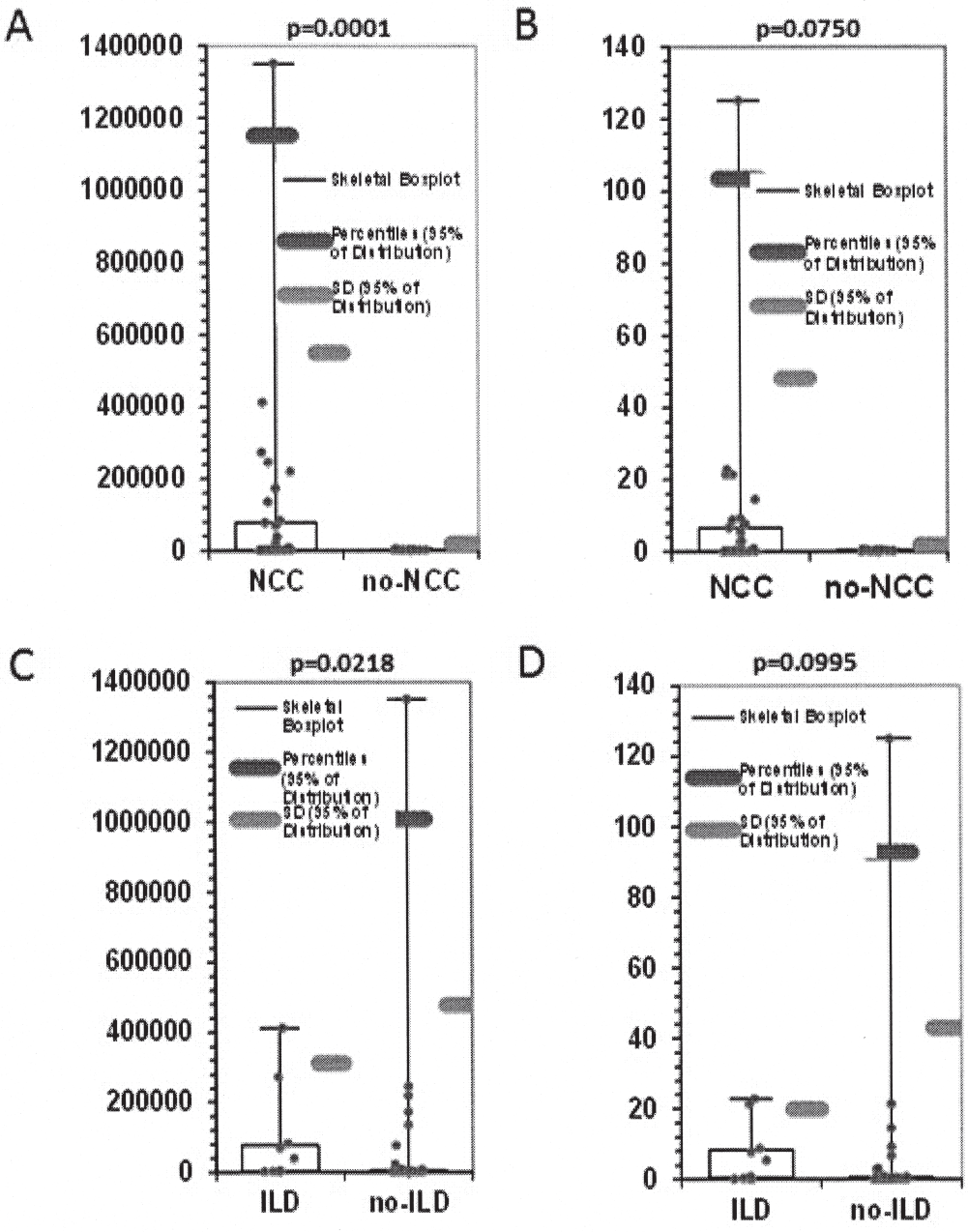


*Figure 4*. Comparative descriptive analysis for clinical associations. Comparative descriptive analyses including median values and interquartile values are shown in panels A and B for nailfold capillary changes (NCC) and in panels C and D for interstitial lung disease (ILD).

different subunits may be associated with different symptoms in SSc.

## DISCUSSION

ANA represent valuable biomarkers in the diagnosis of SSc[1,29], being present in > 90% of the patients. However, because the ANA HEp-2 test has no disease specificity[30,31], serology testing using specific B cell targets is mandatory to confirm positive ANA results and to more accurately characterize patients with SSc. Almost all protein components of the RNase MRP and the evolutionarily related RNase P complex have been reported to be the target of autoantibodies in patients with SARD[10,17]. Studies using ELISA and CLIA confirmed that Rpp25 is a major autoantigen targeted by anti-Th/To antibodies[10,19], being detected in about 60–100% of anti-Th/To reactivity.

Although anti-Th/To antibodies are uncommon in serum samples from patients with SARD, the observation that

*Table 1*. Seroclinical association of anti-Th/To and anti-Rpp25 antibodies.

| | Th/To+ by IP (N = 19) | | | Th/To– by IP (N = 31) | | | p | RP25+ by ELISA (N = 10) | | | RP25– by ELISA (N = 40) | | | p | RP25+ by CLIA (N = 12) | | | RP25– by CLIA (N = 38) | | | p |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | Mean or % | SD or N | Missing | Mean or % | SD or N | Missing | | Mean or % | SD or N | Missing | Mean or % | SD or N | Missing | | Mean or % | SD or N | Missing | Mean or % | SD or N | Missing | |
| Female | 89.5% | 17 | 0 | 80.7% | 25 | 0 | 0.6933 | 80.0% | 8 | 0 | 85.0% | 34 | 0 | 0.6527 | 83.3% | 10 | 0 | 84.2% | 32 | 0 | 1.0000 |
| Age, yrs | 52.4 | 10.7 | 0 | 55.3 | 11.8 | 0 | 0.4530 | 50.3 | 11.7 | 0 | 55.2 | 11.3 | 0 | 0.3625 | 49.6 | 10.7 | 0 | 55.7 | 11.3 | 0 | 0.1166 |
| Meet 1980 ACR classification | 79.0% | 15 | 0 | 80.7% | 25 | 0 | 1.0000 | 80.0% | 8 | 0 | 80.0% | 32 | 0 | 1.0000 | 83.3% | 10 | 0 | 79.0% | 30 | 0 | 1.0000 |
| Disease duration | 11.0 | 10.5 | 0 | 11.2 | 11.4 | 0 | 0.7950 | 10.8 | 9.7 | 0 | 11.3 | 11.3 | 0 | 0.8367 | 9.6 | 9.2 | 0 | 11.6 | 11.5 | 0 | 0.9006 |
| Diffuse disease, % | 10.5% | 2 | 0 | 32.3% | 10 | 0 | 0.1003 | 10.0% | 1 | 0 | 27.5% | 11 | 0 | 0.4155 | 8.3% | 1 | 0 | 29.0% | 11 | 0 | 0.2482 |
| Modified Rodnan skin score (0–51) | 5.7 | 8.9 | 0 | 8.5 | 9.3 | 0 | 0.1982 | 4.3 | 3.6 | 0 | 8.2 | 9.9 | 0 | 0.5419 | 5.0 | 4.1 | 0 | 8.2 | 10.2 | 0 | 0.8819 |
| Raynaud phenomenon | 100.0% | 19 | 0 | 93.6% | 29 | 0 | 0.5192 | 100.0% | 10 | 0 | 95.0% | 38 | 0 | 1.0000 | 100.0% | 12 | 0 | 94.7% | 36 | 0 | 1.0000 |
| Sclerodactyly | 79.0% | 15 | 0 | 80.7% | 25 | 0 | 1.0000 | 90.0% | 9 | 0 | 77.5% | 31 | 0 | 0.6631 | 83.3% | 10 | 0 | 79.0% | 30 | 0 | 1.0000 |
| Calcinosis | 31.6% | 6 | 0 | 19.4% | 6 | 0 | 0.4963 | 20.0% | 2 | 0 | 25.0% | 10 | 0 | 1.0000 | 25.0% | 3 | 0 | 23.7% | 9 | 0 | 1.0000 |
| Esophageal dysmotility | 57.9% | 11 | 0 | 63.3% | 19 | 1 | 0.7034 | 40.0% | 4 | 0 | 66.7% | 26 | 1 | 0.1565 | 41.7% | 5 | 0 | 67.6% | 25 | 1 | 0.1727 |
| Telangiectasias | 73.7% | 14 | 0 | 71.0% | 22 | 0 | 0.8355 | 80.0% | 8 | 0 | 70.0% | 28 | 0 | 0.7042 | 75.0% | 9 | 0 | 71.1% | 27 | 0 | 1.0000 |
| Abnormal nailfold capillaroscopy | 84.2% | 16 | 0 | 62.1% | 18 | 2 | 0.0989 | 100.0% | 10 | 0 | 63.2% | 24 | 2 | **0.0235** | 100.0% | 12 | 0 | 61.1% | 22 | 2 | 0.0101 |
| Digital pits | 42.1% | 8 | 0 | 40.0% | 12 | 1 | 0.8838 | 60.0% | 6 | 0 | 35.9% | 14 | 1 | 0.2794 | 50.0% | 6 | 0 | 37.8% | 14 | 1 | 0.5123 |
| Digital ulcers | 42.1% | 8 | 0 | 41.9% | 13 | 0 | 0.9906 | 50.0% | 5 | 0 | 40.0% | 16 | 0 | 0.7232 | 41.7% | 5 | 0 | 42.1% | 16 | 0 | 1.0000 |
| Interstitial lung disease | 31.6% | 6 | 0 | 19.4% | 6 | 0 | 0.4963 | 50.0% | 5 | 0 | 17.5% | 7 | 0 | **0.0460** | 41.7% | 5 | 0 | 18.4% | 7 | 0 | 0.1292 |
| Pulmonary hypertension | 0.0% | 0 | 1 | 10.0% | 3 | 1 | 0.2819 | 0.0% | 0 | 0 | 7.9% | 3 | 2 | 1.0000 | 0.0% | 0 | 1 | 8.1% | 3 | 1 | 1.0000 |
| Cardiac involvement | | | | | | | | | | | | | | | | | | | | | |
| EF < 50%, % | 0.0% | 0 | 1 | 3.9% | 1 | 5 | 1.0000 | 0.0% | 0 | 0 | 2.9% | 1 | 6 | 1.0000 | 0.0% | 0 | 1 | 3.0% | 1 | 5 | 1.0000 |
| Taking drugs for heart failure, % | 0.0% | 0 | 0 | 9.7% | 3 | 0 | 0.2788 | 0.0% | 0 | 0 | 7.5% | 3 | 0 | 1.0000 | 0.0% | 0 | 0 | 7.9% | 3 | 0 | 1.0000 |
| Taking antiarrhythmics, % | 0.0% | 0 | 0 | 0.0% | 0 | 0 | — | 0.0% | 0 | 0 | 0.0% | 0 | 0 | — | 0.0% | 0 | 0 | 0.0% | 0 | 0 | — |
| FVC, % predicted | 91.5 | 14.6 | 0 | 90.9 | 15.6 | 0 | 0.7490 | 94.3 | 14.8 | 0 | 90.3 | 15.2 | 0 | 0.3889 | 96.3 | 14.5 | 0 | 89.5 | 15.1 | 0 | 0.1426 |
| DLCO, % predicted | 70.7 | 20.9 | 0 | 69.7 | 17.5 | 0 | 0.9124 | 73.7 | 17.3 | 0 | 69.2 | 19.1 | 0 | 0.5046 | 74.3 | 15.7 | 0 | 68.8 | 19.5 | 0 | 0.4133 |
| History of scleroderma renal crisis | 5.3% | 1 | 0 | 3.3% | 1 | 1 | 1.0000 | 0.0% | 0 | 0 | 5.1% | 2 | 1 | 1.0000 | 0.0% | 0 | 0 | 5.4% | 2 | 1 | 1.0000 |
| Proteinuria | | | 2 | | | 3 | 1.0000 | | | 1 | | | 4 | 1.0000 | | | 1 | | | 4 | 1.0000 |
| 0 | 0.0% | 0 | | 96.4% | 27 | | | 0.0% | 0 | | 97.2% | 35 | | | 100.0% | 11 | 0 | 97.1% | 33 | 0 | |
| 1–2 | 0.0% | 0 | | 3.6% | 1 | | | 0.0% | 0 | | 2.8% | 1 | | | 0.0% | 0 | 0 | 2.9% | 1 | 0 | |
| > 3 | 0.0% | 0 | | 0.0% | 0 | | | 0.0% | 0 | | 0.0% | 0 | | | 0.0% | 0 | 0 | 0.0% | 0 | 0 | |
| History of inflammatory myopathy | 0.0% | 0 | 1 | 22.6% | 7 | 0 | **0.0377** | 0.0% | 0 | 0 | 18.0% | 7 | 1 | 0.3186 | 0.0% | 0 | 0 | 18.9% | 7 | 1 | 0.1712 |
| History of arthritis | 16.7% | 3 | 1 | 17.2% | 5 | 2 | 1.0000 | 10.0% | 1 | 0 | 18.9% | 7 | 3 | 0.6673 | 16.7% | 2 | 0 | 17.1% | 6 | 3 | 1.0000 |
| Joint contractures | 10.5% | 2 | 0 | 9.7% | 3 | 0 | 1.0000 | 0.0% | 0 | 0 | 12.5% | 5 | 0 | 0.5687 | 8.3% | 1 | 0 | 10.5% | 4 | 0 | 1.0000 |
| CRP (mg/l) | 6.4 | 11.8 | 3 | 13.1 | 25.1 | 3 | 0.0522 | 5.0 | 8.6 | 1 | 12.1 | 23.4 | 5 | **0.0269** | 4.8 | 7.7 | 1 | 12.6 | 24.0 | 5 | 0.0651 |
| Overlap with other CTD | 0.0% | 0 | 0 | 16.1% | 5 | 0 | 0.1424 | 0.0% | 0 | 0 | 12.5% | 5 | 0 | 0.5687 | 0.0% | 0 | 0 | 13.2% | 5 | 0 | 0.3192 |
| Global assessment of severity (0–10) | 2.1 | 1.6 | 0 | 2.9 | 2.8 | 0 | 0.7196 | 1.7 | 0.7 | 0 | 2.8 | 2.7 | 0 | 0.5585 | 1.7 | 0.7 | 0 | 2.9 | 2.7 | 0 | 0.4416 |
| Global assessments of activity (0–10) | 2.5 | 2.9 | 0 | 2.3 | 2.5 | 0 | 0.8617 | 2.3 | 2.8 | 0 | 2.4 | 2.6 | 0 | 0.9702 | 2.9 | 3.4 | 0 | 2.2 | 2.4 | 0 | 0.5448 |
| Global assessments of damage (0–10) | 2.2 | 1.7 | 0 | 3.2 | 2.5 | 0 | 0.2306 | 2.1 | 1.5 | 0 | 3.0 | 2.4 | 0 | 0.3447 | 2.2 | 1.4 | 0 | 3.1 | 2.5 | 0 | 0.4632 |

P values in bold face are statistically significant. ACR: American College of Rheumatology; CRP: C-reactive protein; CTD: connective tissue diseases; EF: ejection fractions.

*Table 2*. Associations between anti-Rpp25 antibodies (raw data) and clinical data.

| | N | RPP25 ELISA, p | RPP25 CLIA, p | Test |
|---|---|---|---|---|
| History of inflammatory myopathy | 49 | 0.4489 | 0.2139 | Mann Whitney U test |
| Abnormal nailfold capillaroscopy | 48 | 0.0750 | **0.0001** | Mann Whitney U test |
| Interstitial lung disease | 50 | 0.0995 | **0.0218** | Mann Whitney U test |
| C-reactive protein, mg/l | 44 | 0.0762# | **0.0394*** | Spearman correlation |

P values in bold face are statistically significant. #Negative association, Spearman ρ = –0.34; *Negative association, Spearman ρ = –0.28. History of inflammatory myopathy, abnormal nailfold capillaroscopy, and interstitial lung disease were calculated using chi-square test or Fisher's exact test in Table 1.

anti-Th/To antibodies are mostly detectable in SSc makes this specificity an important serological adjunct in the diagnosis of SSc. In addition, current multiplex assays[32] and a screening fluorescence enzyme immunoassay[33] show satisfactory performance characteristics as ANA screening tests for mixed connective tissue disease and Sjögren syndrome, but do not achieve sufficient sensitivity for SSc because of the lack of nucleolar antigens[34]. Consistent with these findings, we also found a high prevalence of nucleolar-positive samples among our ANA-positive/ENA-negative patients, and all anti-Th/To positive samples had a nucleolar pattern. The anti-Th/To test may also have applications to non-SSc patients such as those with ILD because anti-Th/To antibodies have been reported in ~50% of patients with ANA-positive idiopathic pulmonary fibrosis[13].

When the prevalence of anti-Th/To or anti-Rpp25 antibodies in patients with SSc was analyzed, similar prevalences were found: 3.3% (Th/To)[20], 2.1% (Th/To)[21], and 2.9% (Rpp25)[19]. More importantly, the prevalence of anti-Th/To (by IP) and anti-Rpp25 antibodies (by ELISA) was very similar when measured in the same patient cohort[19,35]. However, statistically significant differences in the clinical specificities were found (98.7%[20] and 97.8%[21] for Th/To vs 99.5% for Rpp25[19]). Whether the differences are attributable to the different control groups remains speculative and should be analyzed in future studies.

Although the commercially available LIA contains Th/To subunit based on the hPop1 as the antigen, a significant number (n = 19) of anti-Th/To antibodies (identified by IP) were missed. This may be due to low prevalence of anti-hPop1 antibodies among anti-Th/To–positive patients in our cohort or lack of reactivity with the hPop1 antigen used in LIA. The underlying reason for the lack of correlation of the LIA is most likely due to the different Th/To antigen being used in that assay. About 20% of the anti-Th/To (IP)-positive samples were missed using the Rpp25 assays. The lack of concordance and the potential complementarity of anti-Rpp25 and anti-hPop1 antibodies are currently being studied. In a study by Kuwana, *et al*[17], anti-hPop1 antibodies were significantly more prevalent in anti-Th/To–positive patients with SSc, compared to anti-Th/To–positive patients with other SARD. In contrast, Rpp30 and Rpp38 were equally targeted by antibodies from SSc and non-SSc patients with SARD. Further studies with additional Th/To recombinant or purified proteins are required to verify this finding.

The low prevalence or the absence of several autoantibodies in the ANA-positive/ENA-negative group indicate that the majority of commercially available assays for the detection of autoantibodies have good sensitivity. There were only 2 patients positive for anti-U1-RNP, 2 for anti-topo I[3], 1 for anti-RNAP-III, and none for anti-PM/Scl[6]. In contrast, we found 4 patients with anti-U3-RNP antibodies, of which fibrillarin is a major antigenic target[1]. No conclusions were possible for anti-platelet-derived growth factor because the reported prevalence in SSc is < 5%[2,21]. In addition, 19 anti-Th/To–positive samples were identified. Of interest, 2 sera had antibodies to Su (Ago2)[36] and 1 patient had anti-NOR90 antibodies. Both of those autoantibodies have been reported in various diseases and therefore are not considered SSc-specific antibodies[21,36].

Although known for over 20 years, the clinical association of anti-Th/To antibodies is not fully established. Previous studies are mostly consistent in showing an association with lcSSc; however, association with more specific clinical features are somewhat inconsistent. Small numbers of anti-Th/To–positive patients, differences in ethnicity and environment, differences in the detection methods, recruitment bias, and others could explain the inconsistencies[9,11,35,37,38]. Anti-Th/To antibodies have also been associated with pericarditis and ILD and have a high frequency of "intrinsic" pulmonary hypertension[9,15]. Compared with patients who are anti-CENP–positive, patients with lcSSc who are anti-Th/To–positive have more subtle cutaneous, vascular, and gastrointestinal involvement but more often have certain features typically seen in diffuse SSc, such as pulmonary fibrosis and SSc renal crisis, as well as reduced survival[37]. Like other SSc-related autoantibodies, in patients with Raynaud phenomenon anti-Th/To antibodies are risk factors that are predictive of emerging SSc[39]. Anti-Th/To–positive patients demonstrated earlier development of nailfold capillary microscopy abnormalities than did anti-CENP–positive patients[39]. Anti-Th/To–posi-

tive patients were reported to be younger and more frequently male compared to anti-CENP–positive patients[9]. It has been reported that the prevalence of anti-Th/To antibodies might be higher in white Americans compared to African Americans and Latin Americans[35].

In our cohort of patients with SSc preselected by autoantibody reactivity, we confirmed associations of anti-Th/To and anti-Rpp25 antibodies with ILD and abnormal nailfold capillaroscopy. Our selection criteria to exclude all samples with detectable ANA subspecificities, including non-SSc specific antibodies (such as antichromatin, anti-SSA/Ro60, and anti-Ro/TRIM21 antibodies) and the ANA screening dilution of 1:160 might have introduced a bias into the patient selection. However, this screening dilution was recently recommended by a broad range of experts who used a Delphi approach to achieve consensus[40]. Nevertheless, further studies using the entire CSRG SSc cohort, or patients registered at the European League Against Rheumatism Scleroderma Trials and Research[41] or the German Network for SSc[16], or the Australian cohort[42], are needed to more thoroughly analyze the clinical utility of antibodies to Rpp25.

Despite the low prevalence of anti-Th/To antibodies, testing for those antibodies might have significant value for patient stratification[7,43]. In a previous study, diffuse SSc and lcSSc subsets were associated with particular organ manifestations, but in this analysis the clinical distinction appeared superseded by an antibody-based classification in predicting some SSc-related complications[43].

Autoantibodies to the Th/To autoantigen are important in patients with SSc who have been considered negative for SSc-specific or SSc-associated antibodies by widely available commercial assays. Rpp25 has been confirmed as a major target of these anti-Th/To antibodies. Diagnostic assays for the detection of anti-Th/To and anti-Rpp25 antibodies hold promise to improve the diagnosis and management of SSc.


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## APPENDIX 1.

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