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# Validation of a new immunoenzymatic method to detect antibodies to RNA polymerase III in systemic sclerosis

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## Abstract

### Objective

To test the reliability of a new enzyme-linked immunosorbent assay (ELISA) to identify anti-RNA polymerase III (RNAP III) positive sera from Italian patients with Systemic Sclerosis (SSc) and other chronic inflammatory disorders.

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### Methods

A comparison between the new ELISA for anti-RNAP III and the gold standard technique, immunoprecipitation (IP), was first performed on 106 SSc patients, 16 patients with other connective tissue diseases and 10 healthy subjects. A further ELISA evaluation was performed on 224 SSc patients, 120 subjects with other rheumatic or infectious diseases, and 81 healthy controls.

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### Results

Plotting ELISA and IP data in a Receiver Operator Characteristic curve, the ELISA cut-off value providing the best specificity (99.1%) and sensibility (100%) was 28 U/ml (AUC = 0.999;  $p < 0.0001$ ). Using this cut-off in the second analysis, anti-RNAP III positive results were found in 41 (18.3%) SSc patients, all negative for anticentromere or anti-topoisomerase I antibodies, while only 3 subjects tested positive among the 120 sera collected from other patients. All the healthy subjects were negative.

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### Conclusion

This new ELISA for anti-RNAP III is highly accurate when a proper cut-off value is employed and represents a valid substitute to IP in a clinical setting.

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### Key words

systemic sclerosis, autoantibodies, anti-RNA polymerases antibodies.

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## Introduction

Antibodies to the three (I, II, III) mammalian isoforms of RNA polymerases (RNAP) can be found in patients with autoimmune rheumatic diseases and Systemic Sclerosis (SSc) in particular (1-2). Anti-RNAP positive patients can be divided into three subgroups according to the cross-reactivity shown by their sera with the multiple subunits on the three enzymes (3). The first group is defined by the presence of anti-RNAP I and III; both are rare to be found alone and are highly specific for SSc, especially in its diffuse cutaneous variant (dcSSc) (4). Another group of patients, along with anti-RNAP I-III, can recognize subunits of the isoform II and even this serological reactivity is highly specific for dcSSc. The last subset is composed by patients who result positive for antibodies to the isoform II alone, eventually associated with antibodies to topoisomerase I (topo I) (5). The first two groups are mutually exclusive with respect to anti-centromere (ACA) and anti-topo I, so they can be considered to identify an independent serological subset of SSc patients (6-7). Clinically, reactivity to anti-RNAP I-III or I-II-III has been associated with male sex, an older age of disease onset and a lesser extent of peripheral vascular and pulmonary involvement, but with a higher frequency of cardiac and renal involvement, in particular with the occurrence of scleroderma renal crisis (6,8). The frequency of anti-RNAP I-III in SSc patients shows racial and geographic variations, being higher in Caucasian patients from USA and UK than in patients from Far East and Mediterranean countries (4, 6, 8-11). In Italy, only about 7% of all SSc patients show a positive test for anti-RNAP I-III (10).

The gold standard technique to ascertain the presence of anti-RNAP I-III in patients' sera is immunoprecipitation (IP) using <sup>35</sup>S methionine-labelled HeLa cell extracts as a source of antigen (4). This method requires use of a radioisotope and is not suitable for routine assay or epidemiologic surveys. Recently, Kuwana and coll (12) identified an immunodominant epitope on RNAP III and obtained it in a re-

combinant form for testing SSc sera in an enzyme-linked immunosorbent assay (ELISA) showing high analytical accuracy and specificity (13).

An anti-RNAP III ELISA kit using a recombinant RNAP III protein as antigen source is now ready to be commercialized. In the present paper we tested the reliability of this assay in a selected population of Italian patients with SSc and other chronic disorders.

## Materials and methods

### Patients

This study has been carried out on sera from selected patients followed up in different Centres adhering to the F.I.R.M.A. collaborative group.

All SSc patients have been divided into two groups according to a limited or diffuse cutaneous involvement following the classification suggested by Le Roy *et al.* (14). Median age was 54 years (range 22 to 83) and disease duration 6 years (1 to 30). SSc patients known to be negative for ACA or anti-topo I were preferentially enrolled in order to increase the number of potential anti-RNAP I-III positive cases (5). Control population included patients with other rheumatic diseases (systemic lupus erythematosus; rheumatoid arthritis; Sjögren's syndrome; polymyositis/dermatomyositis; undifferentiated connective tissue disease; seronegative spondyloarthropathies; Wegener's granulomatosis; mixed connective tissue disease), subjects with a positive serologic test for an infectious agents (HIV, HBV, HCV, CMV), and healthy blood donors.

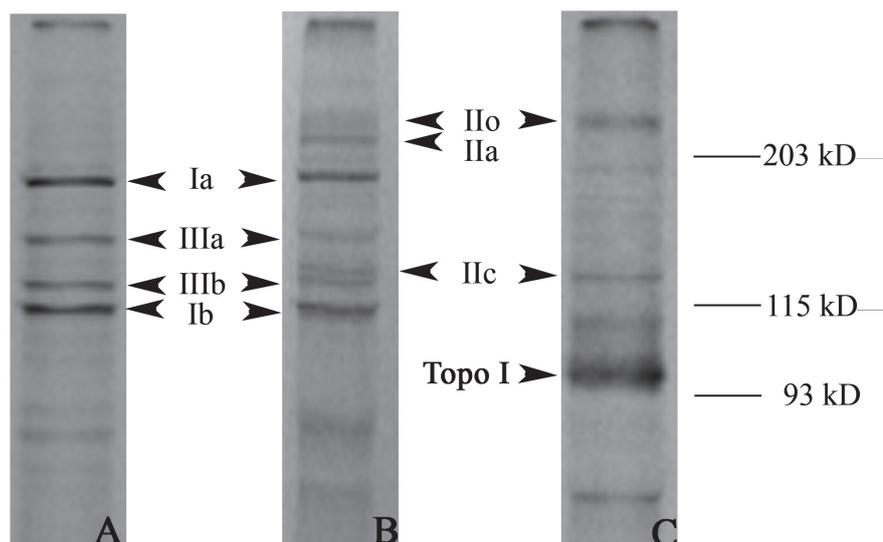
### Routine serologic tests

Antinuclear antibodies (ANA) were detected in all sera by indirect immunofluorescence (IIF) on HEp-2 cells (Diamedix Co, Miami, Florida, USA) with serum diluted 1:80. ACA were identified from the ANA pattern. Anti-topo I were detected by a commercially available ELISA kit (Pharmacia Diagnostic, Freiburg, Germany).

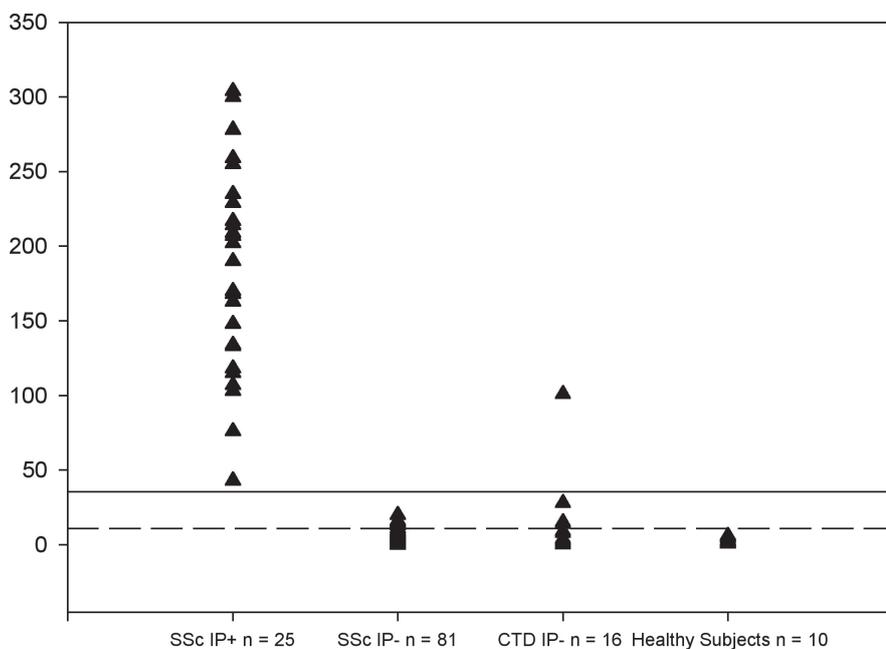
### Anti-RNA polymerases IP assay

Anti-RNA polymerases antibodies have been tested in patients' sera by IP assay with <sup>35</sup>S-methionine labelled HeLa cell

Competing interests: none declared.



**Fig. 1.** In **A**, an immunoprecipitation (IP) profile of a systemic sclerosis (SSc) patient serum recognizing RNA polymerases I-III; in **B**, an IP profile of an SSc patient serum recognizing RNA polymerases I-II-III; in **C**, an IP profile of an SSc patient serum recognizing RNA polymerase II together with topoisomerase I (Topo I). kD = kiloDaltons.



**Fig. 2.** Graph showing all the 132 sera studied both in immunoprecipitation (IP) and ELISA for anti-RNA polymerase III. First column: Systemic Sclerosis (SSc) patients with a positive IP for anti-RNAP I-III or I-II-III. Second column: SSc patients with a negative IP for anti-RNAP I-III or I-II-III. Third column: patients suffering from a connective tissue disease (CTD) other than SSc with a negative IP. Fourth column: healthy subjects. Dashed line shows the 11 U/ml cut-off suggested by the manufacturer. Continuous line indicates the cut-off obtained from the ROC curve, 28 U/ml.

extracts as previously described (10). Briefly, 20  $\mu$ l of undiluted sera were incubated with 0.5 ml of 4 mg/ml protein A-Sepharose CL 4B (Pharmacia, Amersham Biosciences, Uppsala, Sweden) in IP buffer and rotated end-over-end overnight. The antibody-coated protein A beads were then washed 5 times with

IP buffer, centrifuged at 9000 rpm for 1 minute and resuspended in 400  $\mu$ l of IP buffer. Fifteen  $\mu$ l of radiolabeled cell extracts were added to each sample and the samples were rotated end-over-end for 2 hours at 4°C; after 10 washes with IP buffer, the samples were centrifuged at 9000 rpm for 1 minute at 4°C. The

immunoprecipitated proteins were denatured by boiling for 5 minutes in 25  $\mu$ l sample buffer and fractioned in 7.5% polyacrylamide-SDS gels. Gels were then treated with EN<sup>3</sup>HANCE (DuPont NEN, Bruxelles, Belgium) and radiolabeled proteins were visualized by autoradiography. Anti-RNAP reactivities were assessed by comparison with reference samples.

#### Anti-RNA polymerase III ELISA

Anti-RNAP III were assessed by an ELISA kit furnished by MBL (code 7805, Nagoya, Japan). According to the manufacturer's instructions, calibrating solutions and patients' sera diluted 1:100 have been incubated in 96-wells plates coated with the purified recombinant antigen. The bound antibody has been marked with peroxidase-conjugated anti-human IgG goat immunoglobulins, and then incubated with the enzyme substrate. After blocking the peroxidase reaction with a 1N sulphuric acid solution, absorbance was read at a wave-length of 450 nm. For each sample data were collected in duplicate and the optical density (OD) considered was the mean of the two values. Each value has been further converted into U/ml by corrective factors obtained by calibrating solutions ODs.

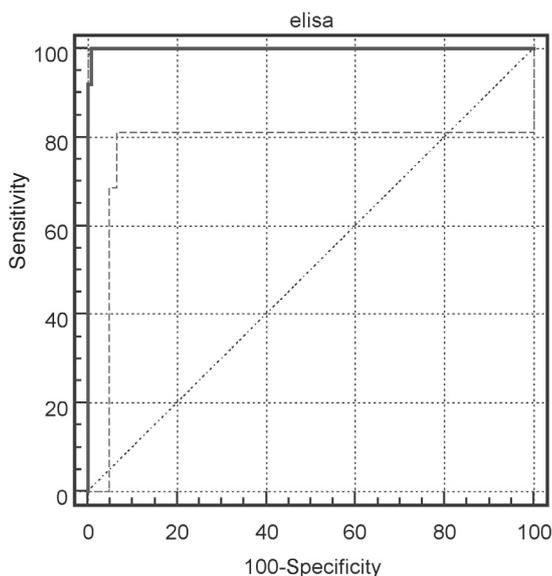
#### Statistical analysis

MedCalc Software (Mariakerke, Belgium) was employed for statistical analysis and the Receiver Operating Characteristic (ROC) Curve. Differences between populations were evaluated by Kruskal-Wallis test. Graphs were obtained by plotting data with SigmaPlot (Systat Software GmbH, Erkrath, Germany).

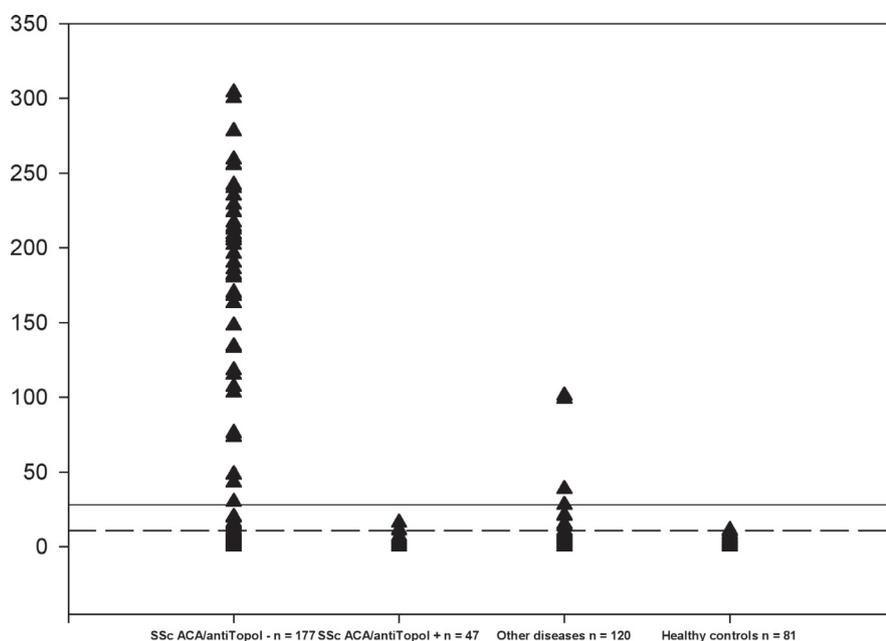
## Results

### IP assay

The presence of anti-RNAP I and III has been assessed by IP in sera from 10 healthy controls, 16 patients with different connective tissue diseases (CTD), and 106 SSc. None of the healthy subjects or CTD patients showed precipitation bands typical of RNA polymerases. The three major anti-RNAP profiles found in SSc patients are shown in Figure 1. Among



**Fig. 3.** The Receiver Operator Characteristic (ROC) curve obtained by comparison of the ELISA results for anti-RNA polymerase III with the gold standard technique, immunoprecipitation. Area under the ROC curve = 0.999, Standard error = 0.004, 95% Confidence interval = 0.971 to 1.000,  $P$  (Area = 0.5) < 0.0001.



**Fig. 4.** Graph showing all the 425 titres obtained in ELISA for anti-RNA polymerase III. First column: anticentromere (ACA) negative and anti-topoisomerase (topo) I negative Systemic Sclerosis (SSc) patients. Second column: ACA positive or anti-topo I positive SSc patients. Third column: patients with other rheumatic or infectious diseases. Fourth column: healthy controls. Dashed line shows the 11 U/ml cut-off, suggested by the manufacturer. Continuous line indicates the cut-off obtained from the ROC curve, 28 U/ml.

these sera, 25 (23.6%) were positive for anti-RNAP I and/or III: 20 co-precipitated RNAP I and III, 4 co-precipitated RNAP I, II and III, and 1 precipitated RNA polymerase III alone. The ANA test of this last patient showed a homogeneous pattern, whilst the anti-RNAP I-II-III positive patient patterns were 1 nucleolar, 1 speckled and 2 with a combination of these two patterns. As for patients with anti-RNAP I-III, one had

negative ANA test, 10 had a speckled pattern, 4 nucleolar, 3 homogeneous, and 2 had speckled and nucleolar. Anti-RNAP I-III or I-II-III were found in 18 out of 47 (38.3%) patients with diffuse cutaneous involvement and in 7 out of 59 (11.9%) with limited cutaneous SSc. All of the ACA positive sera (10 cases) and anti-topo I positive sera (17 cases) were negative for anti-RNAP I-III or I-II-III.

*Comparison between IP and ELISA for cut-off value definition*

The results obtained by ELISA in the patients also studied by IP are reported in Figure 2. Using the cut-off suggested by the manufacturer (11 U/ml), all of IP positive sera were positive by ELISA and all of 10 healthy controls were negative. However 10 out of 81 (12.3%) IP negative SSc sera as well as 4 out of 16 (25%) IP negative CTD sera were positive by ELISA.

Considering IP as the gold standard technique, we obtained a ROC curve (Fig. 3). By raising the cut-off value from 11 to 28 U/ml, the ELISA specificity could be improved from 88.8% to 99.1% keeping a sensitivity of 100% (area under the curve (AUC) = 0.999; Standard error = 0.004; 95% Confidence interval = 0.971 to 1.000,  $p$  < 0.0001). In fact, only those 25 SSc patients who were anti-RNAP I-III or I-II-III positive by IP had a titre over the new cut-off value, whilst only 1 patient among the CTD group still had a positive anti-RNAP III titre in ELISA. This last patient was suffering from subacute cutaneous lupus.

*ELISA analysis of a large series using the newly defined cut-off value*

ELISA was then performed on 81 healthy control sera, 120 sera from patients with different rheumatic or infectious diseases and 224 sera from SSc patients, using 28 U/ml as the cut-off value.

As shown in Figure 4, all the healthy subjects were negative. Among the 120 sera collected from patients with other rheumatic or infectious disorders, only 3 (2.5%) resulted positive: one patient with systemic lupus erythematosus, one with Sjögren's syndrome and one with a positive HIV serology. As for SSc, 41 out of 224 (18.3%) sera were positive for anti-RNAP III, with ANA test being positive in all but one of them. Nineteen out of 40 (47.5%) had a speckled pattern, 8 (20%) nucleolar, 5 (12.5%) homogeneous and 8 (20%) speckled and nucleolar. Anti-RNAP III were found in 32 out of 103 (31.1%) patients with diffuse cutaneous involvement and in 9 out of 121 (7.4%) with limited cutaneous SSc ( $p$

< 0,001). All of ACA positive sera (28 cases) and anti-topo I positive sera (46 cases) were negative for anti-RNA pol III (Fig. 4).

Clinical sensitivity and specificity for SSc were 15.4% and 98.5% respectively.

Finally, we analyzed the discriminating power of this anti-RNAP III test to identify SSc from the two control populations. There was a statistically significant difference in anti-RNAP III positivity between SSc patients and healthy controls ( $p < 0.001$ ) and also between SSc and CTDs ( $p < 0.05$ ), while the test was not significant in discriminating CTDs from healthy subjects.

### Discussion

So far, large studies on anti-RNAP I-III distribution and associations have been contrasted by the lack of available routine tests (9-10). Anti-RNAP I-III have been included among the heterogeneous group of anti-nucleolar autoantibodies that, together with ACA and anti-topo I, account for a positive ANA test in more than 90% SSc patients (15). However, only the isoform I of the enzyme has a nucleolar localization and therefore shows a nucleolar pattern when recognized by its autoantibody counterpart in IIF. RNAP III resides in the nucleus and gives a speckled and/or homogeneous pattern in IIF (1). Thus, IIF is not a specific assay to detect the presence of anti-RNAP I-III, and in other common techniques as immuno-blotting or counter-immunoelectrophoresis, employed to identify several extractable nuclear antigens, the antibody has always shown a poor sensitivity (3).

The data shown by the present study provide evidence of how this new ELISA may be accurate in identifying anti-RNAP III in patients' sera, representing, after reconsideration of its cut-off, a valid substitute to the IP technique. As a semi-quantitative test, easy to perform on large populations and on serial samples, it may be very important for predicting the course of one of the most serious SSc visceral involvement, scleroderma renal crisis. It has been shown indeed that the development of this condition is associated with anti-

RNAP III and that it can be preceded by a rapid increase of antibody titre (13). This issue could give its contribution to an early diagnosis and then to a prompt and aggressive treatment of this severe and life-threatening SSc complication, thus leading to a dramatic improvement of its outcomes (16).

The frequency of occurrence of anti-RNAP III and their clinical associations are beyond the aim of the present study. In particular, it should be noticed that our SSc patients have been preferentially enrolled if known to be ACA or anti-topo I negative or known to be anti-RNAP III positive by IP (10). This point has to be taken into account when interpreting the reported frequency of anti-RNAP III in the present study since it does not reflect the actual prevalence in Italian SSc patients, which is likely to be much lower (10).

Beyond the inclusion criteria of patients, this study confirms that, after defining a proper cut-off value, anti-RNAP III detected by ELISA are highly specific for SSc, mutually exclusive with respect to the other major serologic subsets, i.e. ACA and anti-topo I, and strongly associated with a diffuse cutaneous involvement. Also, the low ELISA sensitivity reflects the low frequency of anti-RNAP III in SSc patients from our country (10). In other words, the results obtained by ELISA are almost identical to those obtained by IP. This is of particular interest since ELISA usually shows a markedly superior cost-effectiveness with respect to a more sophisticated and time consuming technique like IP (12).

A commercially available test for anti-RNAP III can indeed make studies on large series easier, with all the consequences this implies in a disease whose diagnosis and prognosis is so greatly related to autoantibody profile like SSc is (17). This is particularly important in countries where the frequency of these autoantibodies is low (9-10), and extensive surveys are needed to identify unequivocal clinical associations.

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