Detection of anti-mitochondrial antibodies by immunoprecipitation
in patients with systemic sclerosis

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ABSTRACT.

OBJECTIVE. To describe a new immunoprecipitation pattern identified in Italian patients affected by systemic sclerosis (SSc), corresponding to the pyruvate dehydrogenase antigen complex recognized by anti-mitochondrial antibodies (AMA) in primary biliary cholangitis (PBC).

METHODS. Autoantibodies in sera from 85 patients with SSc were tested by protein- and RNA-immunoprecipitation. Immunoprecipitation-Western blot was used to determine the identified proteins, and medical records re-evaluated for liver function tests and PBC.

RESULTS. In 13/85 (15%) SSc sera, a unique set of 75-50-40-34kd proteins that had not been previously reported, was noted. The four proteins were identified as the proteins X/E3BP, E1α, E1β, and E2/E3 of the pyruvate dehydrogenase antigen complex by immunoprecipitation-Western blot. From clinical record evaluation, 9/13 (69%) SSc patients with this new pattern were positive for AMA by routine indirect immunofluorescence, and 7/13 (54%) had a diagnosis of PBC, while 4/13 (31%) manifested no biochemical signs of cholestasis. Twelve of 13 patients with SSc and AMA by immunoprecipitation have a limited cutaneous form of SSc and anti-centromere antibodies.

CONCLUSIONS. We describe a pattern of 4 proteins in 15% of SSc patients, identified for the first time by protein-immunoprecipitation. This pattern corresponds to serum AMA against the pyruvate dehydrogenase antigen complex and it must be considered in the interpretation of protein-immunoprecipitation results.

Key words: autoimmunity; scleroderma; autoimmune cholangitis; pyruvate dehydrogenase; immunoprecipitation
INTRODUCTION

A large proportion of patients with systemic sclerosis (SSc) manifest a coexisting autoimmune condition, including primary biliary cholangitis (PBC) [1, 2]. As immunoprecipitation (IP) is currently one of the most sensitive techniques to detect new and known rare autoantibodies, a clear understanding of common patterns is necessary for proper interpretation of the results. Anti-mitochondrial autoantibodies (AMA) are the hallmark of PBC [3-5] and in clinical practice they may predate the clinical onset of disease [6, 7] when tested with routine indirect immunofluorescence performed on tissue slides, while the use of alternative techniques such as ELISA for mitochondrial antigens still is not routinely used and it has unclear clinical significance [8]. PBC is associated with other autoimmune diseases in about 30% of patients, with SSc found in 7-12% of cases [1, 2], even though in clinical practice a higher percentage of SSc patients may have biochemical liver abnormalities without clinical significance [9].

We herein used IP to test new and uncommon serum autoantibodies in SSc and in this screening analysis we observed that 15% of SSc sera manifest a novel IP pattern, comprising a set of 4 proteins corresponding to the E1α, E1β, protein X/E3BP, and E2/E3 subunits of the pyruvate dehydrogenase complex (PDC) recognized by AMA [10-12]. The prevalence of AMA by IP outnumbers what is observed in routine tests in our cohort of SSc patients and this new IP pattern should be known when interpreting IP data in SSc sera.
PATIENTS AND METHODS

Subjects.

Eighty-five consecutive patients with SSc attending the outpatient Rheumatology clinic at Humanitas Research Hospital (Rozzano, Milan, Italy) between 2012 and 2016, were enrolled to the study. Controls included sera from 74 healthy subjects, 49 patients with polymyositis/dermatomyositis (PM/DM), 32 patients with undifferentiated connective-tissue disease (UCTD), and 2 patients with established PBC previously AMA-positive and negative for serum antinuclear antibody (ANA) without signs of rheumatic diseases. Internationally accepted criteria were used for the diagnosis of SSc [13], PM/DM [14], UCTD [15], and PBC [16] and we collected clinical and laboratory data at enrollment. SSc patients who did not fulfill the ACR/EULAR criteria were defined as sine SSc [17] and very early diagnosis of SSc (VEDOSS) [18]. Liver laboratory tests included in our study are aspartate aminotransferase (AST), alanine aminotransferase (ALT), gamma glutamyltransferase (gammaGT), alkaline phosphatase (ALP), bilirubin (total, direct) and, when available, liver histology was also evaluated. The study was approved by the Institutional Review Board of the Humanitas Research Hospital and a signed informed consent was obtained from all subjects in accordance with the Declaration of Helsinki and its subsequent modifications.

Methods.

Protein- and RNA-immunoprecipitation (IP)

Sera were obtained from whole blood through centrifugation at 2000g for 15 minutes, and then stored in -20°C freezer until use. Serum autoantibodies were screened by protein-IP using 35S-methionine-labeled K562 cell extract followed by SDS-PAGE and autoradiography, and by RNA-IP using unlabeled K562 cell extract followed by urea-PAGE and silver staining [19, 20]. Autoantibodies were analyzed using reference sera obtained from the Autoantibody Standardization Committee (www.autoab.org) and from internal controls, and they were used for the correct
interpretation of protein-IP bands for known ANA specificities. These reference sera help in
determining protein-IP specificities for ANA in connective tissue diseases and are used also for SSc
patients as in our cohort.

**IP-Western Blot (IP-WB)**

Sera with a novel IP pattern of a set of 4 proteins were tested by IP-WB. In detail, 50µl of candidate
sera were cross-linked with protein-A Sepharose beads and then immunoprecipitated with cell
extract from 5x10⁶ K562 cells/sample. Proteins were then fractionated by 8% SDS-PAGE and
transferred to a nitrocellulose filter, probed with 1:500 of mouse polyclonal anti-human PDH E1α
antibody (Novus Biologicals, Littleton, CO, USA) for a 41 kD protein identification, followed by
horseradish peroxidase (HRP) goat anti-mouse IgG (1: 10 000 dilution; ThermoFisher, Waltham,
MA, USA). The same procedure was used to identify the other bands of the complex: mouse anti-
human PDH E1β (1: 500 dilution; Novus Biological, Littleton, CO, USA) for the protein of 34 kD;
mouse anti-human PDH protein X/E3BP (1: 1000 dilution; Novus Biological, Littleton, CO, USA)
for the 54kD; mouse anti-human PDH E2/E3 proteins of 58kD and 74kD (1: 10,000 dilution;
Abcam, Cambridge, UK) followed by goat anti-mouse IgG (ThermoFisher, Waltham, MA, USA).
Development was performed by Immobilon Western Chemiluminescent HRP substrate (Millipore,
Darmstadt, Germany) and acquired using ChemiDoc (Bio-Rad, California, USA).

**Indirect immunofluorescence (IIF)**

Antinuclear and cytoplasmic antibodies were tested by IIF on HEp-2 ANA slides (INOVA
Diagnostics, San Diego, CA, USA) using a 1:80 dilution of human sera of patients and controls,
followed by AlexaFluor488 AffiniPure F(ab’)2 fragment goat anti-human IgG, Fcγ fragment
specific (Jackson Immunoresearch Europe Ltd, Suffolk, UK) as previously described [19]. Images
were acquired on Olympus BX53 Upright fluorescence microscope. No immunofluorescence
analysis on tissue slides was performed for AMA identification.
ELISA

Positive serum AMA identified by protein-IP (n=13) were tested for confirmation by ELISA using the QUANTA Lite M2 EP (MIT3) IgG ELISA (INOVA Diagnostics, Inc, San Diego, USA) with the patented pMIT3 antigen, which consists of a recombinant protein containing the immunodominant epitopes of the 3 major AMA targets [21], currently limited to research settings. ELISA was performed according to the manufacturer instructions, and results were shown as Units.

Statistical analysis

All comparisons were performed by Fisher’s exact test using Prism version 5.0 (GraphPad Software, Inc., La Jolla, CA, USA). All analyses were two-tailed and P values <0.05 were considered as statistically significant.
RESULTS

Demographic and laboratory data of the SSc cohort.

We included 85 patients (81 women, mean age ± standard deviation 66±15 years) with SSc and their sera were studied by IP. The age at SSc onset was 53 ± 15 years, and the mean follow-up was 106 months (range 4 to 408). From clinical record retrospective evaluation, nine patients with limited cutaneous SSc had a previous diagnosis of PBC with available liver biopsy, and in one patient an overlap with ANA-positive autoimmune hepatitis was found. In 5/9 (56%) PBC cases, the onset of chronic cholestasis preceded the diagnosis of SSc (range 1-24 years), while in 4/9 (44%) the diagnosis of PBC followed SSc by 3-24 years. Additional features of our SSc cohort are described in Table 1. Fourteen cases defined as sine SSc [17] (n=4) and very early diagnosis of SSc (VEDOSS, [18], n=10) are not included in the statistical evaluation shown in Table 1, but two AMA-positive IP cases were identified in sine SSc patients defined by ACA-positivity, Raynaud’s phenomenon and capillaroscopy alterations but no signs of skin fibrosis.

Autoantibody analysis.

By protein-IP we observed a new pattern with a set of 4 proteins of 75-50-40-34kd molecular weight (Figure 1) in 13/85 (15%) SSc sera, while no RNA band was detected by RNA-IP in these patients. The protein-IP pattern was observed also in the two AMA-positive PBC cases, and in one patient with UCTD without sign of liver disease suggestive for PBC, while no PM/DM manifested such pattern. We could identify each protein immunoprecipitated by these sera using IP-WB (Figure 2) as the E1α, E1β, E2/E3 and protein X/E3BP subunits of the mitochondrial pyruvate dehydrogenase complex.

The main features of SSc cases with the new protein-IP profile are summarized in Table 2, and a comparison between AMA-positive and AMA-negative limited cutaneous SSc patients is described in Table 3. From a clinical viewpoint, 8/13 (61.5%) SSc patients with the AMA protein-IP profile we describe for the first time were positive for serum AMA by routine IIF on tissue slides, while
5/13 (38.5%) SSc patients tested negative and had no cytoplasmic staining was reported by routine IIF on Hep-2 slides; among these, 6/13 (46%) had a pre-existing diagnosis of PBC. In our cohort AMA-positive SSc patients have a limited cutaneous form and they are significantly associated with ACA positivity (*Table 3*), and only one AMA-positive case has diffuse cutaneous SSc.

The pMIT3 ELISA results were in agreement with the new protein-IP profile in 13/13 (100%) sera but we should note that this serology method is currently used only for research purposes. ACA-positive IIF pattern with cytoplasmic staining at different titer is present in 12/13 (92.3%) AMA-positive SSc cases, and in one case we had anti-cytoplasmic, nuclear speckled+nucleolar pattern by IIF (*Figure 3*).
DISCUSSION.

Serum autoantibodies remain the most commonly used and helpful biomarker in the diagnosis and treatment of autoimmune diseases and may precede the clinical manifestations by several years, as reported in systemic lupus erythematosus [22] and rheumatoid arthritis [23]. In some cases, however, serum autoantibodies such as ANA are detected in non-rheumatic conditions or may be falsely positive due to technical issues, particularly at low titer [24], while other autoantibodies have strong clinical associations such as anti-topo I/ScI70 being frequently associated with diffuse cutaneous SSc [25]. Indeed, PBC and SSc represent unique conditions because they often coexist despite being considered rare [1] and they manifest serum autoantibodies with high specificity, represented by AMA and ACA, respectively.

The diagnosis of PBC in patients with rheumatic diseases may be challenging until the clinical and laboratory expression of the disease, although it has been suggested that AMA-positive asymptomatic subjects will eventually develop PBC and that an early use of ursodeoxycholic acid may improve prognosis [26]. The serological scenario of SSc is further complicated by the recent description of several rare autoantibodies associated with SSc [27]. These new autoantibodies are identified first by IP in research laboratories and then validated by other techniques to be ultimately used in clinical practice, as in the case of recently identified autoantibodies in PM/DM [28]. Based on this rationale, identifying and sharing common patterns by IP is necessary to allow the testing of new and rare autoantibodies, and to improve the interpretation of IP results.

We herein report for the first time the IP pattern of serum AMA in SSc and the prevalence of PBC-associated autoantibodies even in the absence of biochemical sign of cholestasis, through the identification of a set of 4 proteins that correspond to the subunits of the pyruvate dehydrogenase complex. The conventional pattern of AMA detected in sera from PBC was first described by techniques such as immunoblot and showed the reactivity of PBC sera with components of the PDC complex, namely E1α/β, E2, E3 and the protein X later identified as E3BP [29]. The novelty of our finding is based on the description for the first time of a protein-IP pattern that corresponds to the
antigenic subunits recognized by AMA, which has never been described before. This identity of the proteins identified in this new protein-IP pattern was confirmed using the currently available ELISA, yet not routinely used, i.e. the recombinant pMIT3 antigen [21], and the IP-WB technique. It is possible that AMA positivity we observed by protein-IP may also include the reactivity only of some of the presented bands but this was not observed in our series. Further, we report that 15% of SSc sera are positive for AMA, a significant prevalence as demonstrated also by previous reports with up to 25% AMA positivity in SSc patients [8], and ACA are reported in up to 30% of patients with PBC [30].

Our data demonstrate that one laboratory technique, protein-IP, could be sufficient to achieve two goals: to screen for autoantibodies in rheumatic patients such as SSc and to identify coexisting autoantibodies that discriminate comorbidities. Moreover, we are currently living in an era characterized by the use of automatic screening method for ANA identification that often do not include the cytoplasmic pattern in AMA-positive patients, thus we could overcome this problem by using IP alone. Nonetheless, we are aware that protein-IP has a limited feasibility in the routine laboratory due to the labor and time-consuming protocols and the significant costs.

Four patients with SSc and the new protein-IP pattern had no sign of PBC nor tested AMA positive by IIF and this is particularly intriguing as the use of pMIT3-based ELISA has been shown to reduce the proportion of AMA-negative PBC cases and was in 100% agreement with protein-IP, confirming the identity of the protein pattern we observed [31]. Our results have three possible implications. First, we may speculate that AMA detected by protein-IP are more sensitive and possibly appear earlier than IIF-AMA on tissue slides, and they could be recognized in an early phase of autoantibodies screening. Second, previous studies on the coexistence of PBC and SSc may have underestimated the prevalence of the association, as AMA are usually detected when a clinical or laboratory suspect of PBC is present [8]. We are well aware that AMA positivity in the absence of biochemical cholestasis should be further investigated by liver histology to confirm the presence of PBC but this is currently not advisable for ethical reasons nor a diagnosis at
asymptomatic stages would change the natural history of PBC despite the use of ursodeoxycholic acid [32]. Third and last, we confirm that AMA and PBC are associated with the limited cutaneous form of SSc and positivity of ACA, and liver disease does not seem to worsen the prognosis of our AMA-positive patients with SSc [2].

In conclusion, we describe for the first time the protein-IP pattern of AMA and we demonstrate for the first time that protein-IP can be used for AMA detection in our SSc cohort of patients. In 15% of our SSc cases, in fact, a new AMA IP pattern was detected and characterized as a set of 4 proteins corresponding to the subunits of the pyruvate dehydrogenase antigen complex, as described decades ago only by immunoblot [29]. The definition of this protein-IP pattern is mandatory in the interpretation of protein-IP, increasingly used for rare autoantibody detection in clinical practice.

Ultimately, we could also speculate that IP-AMA are earlier predictors of PBC compared to routine AMA on tissue slides, similar to the predictive role of AMA in patients without cholestasis, but this needs to be verified in a larger prospective cohort.

ACKNOWLEDGEMENTS.

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TABLE 1. Main features of the limited and diffuse cutaneous SSc cohort analyzed for AMA identification by protein-IP. Only p values <0.05 are reported. Fourteen cases defined as sine SSc (n=4) and VEDOSS (n=10) are not included in the present table.

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<th>Diffuse cutaneous SSc (n=12)</th>
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<td>50 (6)</td>
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<td>Anti-RNAPIII, % (n.cases)</td>
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<td>8 (1)</td>
<td>ns</td>
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Abbreviations: ACA= anti-centromere antibodies; AMA= anti-mitochondrial antibodies; ANA=anti-nuclear antibodies; IP= immunoprecipitation; PBC=primary biliary cholangitis; RNAPIII= RNA polymerase III
TABLE 2. Demographic, clinical and laboratory features of SSc patients with detectable AMA by protein-IP.

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**Abbreviations:** ACPA= Anti cyclic-citrullinated peptide antibodies; ALP= alkaline phosphatase; ALT= alanine aminotransferase; aPLs= anti-phospholipid antibodies; AIH= autoimmune hepatitis; AMA= anti-mitochondrial antibodies; ANCA= anti neutrophil cytoplasmic antibodies; AST= aspartate aminotransferase; AZA= azathioprine; CTX=cyclophosphamide; D= diffuse SSc; gammaGT= gamma glutamyltransferase; IIF= indirect immunofluorescence; L= limited SSc; MTX= methotrexate; n/a= not available; PDN= prednisone; SSc= systemic sclerosis; UDA= ursodeoxycholic acid.
Table 3. Comparison of main clinical and laboratory features in patients with limited cutaneous SSc based on serum AMA IP positivity. Two AMA-positive cases sine SSc and one AMA-positive diffuse SSc patient are not included in the present table. Only p values <0.05 are reported.

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REFERENCES.


FIGURE LEGENDS.

Figure 1. IP pattern of AMA-positive patients. The protein-IP pattern of the 75-50-40-34kD proteins (black arrows) of the mitochondrial complex antigen is shown in 13 SSc patients. IP results in 8% SDS-PAGE gel are shown. PBC #1 and PBC #2 represent two cases with isolated diagnosis of PBC and PBC #2 was known to have weak positivity for AMA by routine IIF on tissue slides and ELISA. Normal human serum is included as negative control.

Figure 2. IP-WB confirming the identity of the IP bands corresponding to the mitochondrial antigen complex components. The samples tested by IP-WB are the same shown in Figure 1 for protein-IP, with positivity for the IP-AMA pattern as described, and their main features are described in Table 2. The molecular weight marker (MWM) is shown for reference, and IP-WB for each component confirms the identity of protein X/E3BP, E1α, E1β and E2/E3 corresponding to the 54kD, 41kD, 34kD, 74/58kD of the proteins shown in protein-IP, respectively.

Figure 3. Indirect immunofluorescence of AMA-positive SSc cases detected by protein-IP. HEp-2 ANA slides were used to analyze the immunofluorescence pattern of AMA-positive SSc cases (a to m), or normal human serum (n). Serum dilution, 1:80. ACA-positive with cytoplasmic staining at different titer is present in 12/13 AMA-positive SSc cases (a to l), and in one case we had a cytoplasmic staining associated with positive anti-nuclear and nucleolar immunofluorescence staining (m).