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# Protein and RNA immunoprecipitation for the identification of specific serum autoantibodies in systemic autoimmune rheumatic diseases.

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## ABSTRACT

Serum autoantibodies play a key role in systemic autoimmune rheumatic diseases for diagnostic, classification and prognostic purposes. Research of new autoantibodies has been very active in the last decade in rare connective tissue diseases such as systemic sclerosis and poly/dermatomyositis, with new biomarkers entering the clinical practice. Immunoprecipitation of protein and/or RNA components of the target autoantigens constitutes the gold standard method for the discovery of new autoantibodies in a screening setting but is considered time- and labor-intensive and, accordingly, is performed only in a few laboratories worldwide. As a result, alternative techniques such as ELISA and immunoblotting are often preferred for large-scale testing, despite the lack of standardization.

The aims of the present project are (1) to set up protein- and RNA- immunoprecipitation in our laboratory and (2) to describe serum autoantibodies identified in our series of patients affected by systemic autoimmune rheumatic diseases. During the PhD program we were able to perform correctly protein-and RNA-immunoprecipitation in our laboratory as demonstrated by positive reference sera, and then by the identification of known but also new and rare autoantibodies, as represented by two new patterns immunoprecipitated in systemic sclerosis, corresponding to serum anti-hnRNP-L and anti-mitochondrial antibodies. In psoriatic arthritis we also analyzed the concentration of circulating levels of LL37, a recently established target of autoimmune response at the skin level, and we identified an increased production in a subset of patients. In conclusion, performing protein- and RNA-immunoprecipitation as a screening method in our laboratory allows a more complete and specific autoantibody analysis that cannot be performed by the commercial techniques available nowadays, and further analysis of the role of LL37 in psoriatic arthritis patients may help in the identification of a new biomarker in this condition.

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## INTRODUCTION

### 1. AUTOANTIBODIES IN SYSTEMIC AUTOIMMUNE RHEUMATIC DISEASES.

Systemic Autoimmune Rheumatic Diseases (SARDs) include chronic conditions characterized by the presence of specific serum autoantibodies (autoAbs) and a panel of symptoms related to the autoimmune injury of joints, skin and internal organs. The clinical expression of SARDs can be very heterogeneous, and they can be divided into three main groups, as shown in **Figure 1 panel A**. Among SARDs, Connective Tissue Diseases (CTDs) refer to chronic inflammatory and autoimmune conditions that affect the connective tissue, and are characterized by a non-organ specific autoimmune reaction characterized by serum autoAbs [1, 2]. This group includes Systemic Lupus Erythematosus (SLE), Sjögren's Syndrome (SjS), Systemic Sclerosis (SSc), Poly/Dermatomyositis (PM/DM), and some forms that do not fulfill the established diagnostic criteria called undifferentiated connective tissue disease (UCTD) [3, 4], while the presence of multiple CTDs (SLE, SSc and PM) in the same patient with positive anti-U1RNP antibodies is defined mixed connective tissue disease (MCTD) [5]. As for arthritis, we can divide them in two main groups: the first one is represented by Rheumatoid Arthritis (RA), a chronic inflammatory polyarthritis affecting synovial joints (and more rarely internal organs) that is characterized by positivity of Rheumatoid Factor (RF) and anti-cyclic citrullinated peptide (CCP) in up to 70% of patients, and for this reason an RA patient can be called seropositive; the second group is represented by seronegative arthritis such as psoriatic arthritis (PsA), ankylosing spondylitis and reactive arthritis that are negative for RF and anti-CCP antibodies as required in the diagnostic criteria [6]. The third group of SARDs is represented by vasculitidies, conditions characterized by the inflammation of small, medium or large vessels, in which the only biomarker available for their diagnosis is represented by ANCA (anti-neutrophil cytoplasmic antibodies), positive in a reduced number of cases represented by eosinophilic granulomatosis with polyangiitis, granulomatosis with polyangiitis and microscopic polyangiitis [7].

The diagnosis of CTDs relies not only on clinical symptoms and signs, but also on the identification of autoAbs that are associated with specific clinical manifestations and for this reason they are also called disease-marker antibodies and they are so important that they are part of the diagnostic criteria for some of these CTDs (**Figure 1, panel B**) [1, 2, 8]. The importance of autoAbs relies also on the fact that they can be present years before the clinical manifestations of SARDs, and they can be used also for their predictable value as they can characterize early and undifferentiated autoimmune diseases [8]. For example, the positivity of anti-nuclear antibodies (ANA) in the presence of a symptom such as Raynaud's phenomenon is part of the criteria for very early diagnosis of SSc, and this early diagnosis can change the follow-up, prognosis and therapeutic approach in SSc patients [9].

As shown in **Figure 1 panel B**, many known autoAbs are used as routine clinical tests for diagnostic and classification purpose, but many new autoAbs are not part of standard laboratory tests and more still need to be identified. In fact, in recent years there has not been any identification of new autoAbs in SLE and SSc with significant impact on clinical medicine, while several new autoAbs have been identified in PM/DM patients but they can be searched only in research and not in routine settings [10].

*1.1- Autoantigens targeted by autoantibodies.*

Antigens recognized by autoAbs are self-antigens first characterized in 1948 with the LE cell phenomenon described as the engulfment of denatured nuclear material of a cell by a neutrophil or macrophage [11]. In 1976 anti-Sm antibodies were identified in patients affected by SLE [11], and in the following years thousands of cellular proteins have been identified but only a few decades of autoantigens (autoAgs) have been described in SARDs. AutoAgs are usually macromolecular particles defined as multiprotein or nucleoprotein complexes with important functions in the cells such as RNA splicing and chromatin organization [12]. DNA-protein autoantigens include the histones/chromosomes, NOR90, centromeres, Ku antigen, while RNA-protein complex autoAg are UsnRNPs (U1RNP, Sm antigens), U3/fibrillarin and Th/To. These complexes tend to be localized

in different subcellular compartments and this is the reason why they appear in specific patterns at immunofluorescence, as for the nucleolar staining in SSc autoantibodies [10, 11].

The autoimmune response directed against autoAgs takes place through multiple ways and it is triggered by an abnormal processing of self-antigens by antigen-presenting cells (APCs). The presence of altered protein-protein or protein-nucleic acid interactions and the somatic mutation of self-antigens (such as p53) may trigger the immune system through activation of autoreactive T cells and production of autoAbs that may spread within the molecule or other components [10, 11]. The presence of altered patterns of protein degradation such as abnormal protease expression or apoptosis pathways can enhance the presentation of cryptic peptides to autoreactive T cells [10]. These autoimmune mechanisms are very selective because only a limited numbers of proteins are seen by IP using radiolabeled cell extract that contains thousands of proteins. In addition, in SSc and PM/DM presence of more than one disease-marker autoAb is uncommon: they are mutually exclusive and they do not change overtime despite a variation in the clinical features of the autoimmune disease.

In conclusion, we can say that the exact mechanism leading to autoAb production and manifestation of disease in the form of SARDs is unknown, but several hypothesis suggest that a possible trigger of the autoimmune response could be a quantitative or qualitative change in protein, DNA or RNA structure, and formation of cryptic epitopes expressed by APCs that are responsible for no or incomplete tolerance and can trigger an autoimmune response [13, 14].

*1.2- Anti-nuclear (ANA) and ENA (extractable nuclear antigens) antibodies.*

Serum ANA are autoAbs directed against self-antigens that are produced in systemic autoimmune conditions, or also in the presence of organ-specific autoimmune diseases (i.e. autoimmune thyroiditis), cancer or infections [1, 12, 15]. The standard method used test for the identification of ANA is indirect immunofluorescence (IIF) [16], which is routinely performed in the laboratories worldwide and allows the identification of the ANA pattern and titer. The nuclear staining patterns are usually classified into speckled, homogeneous, nucleolar and centromere and reported routinely

[16], but there are other less frequent patterns that can be seen only in a limited number of patients. As for the titer, ANA positivity ≥1:160 is considered clinically significant by most routine autoimmunity laboratories and strong positivity is associated with the presence of autoimmune disorders, while titers ≤1:160 are present in up to 20% of healthy people, mainly in the elderly and in female population [17] as we also recently demonstrated in a study on ANA prevalence and risk of SARDs in a specific area of Northern Italy [18]. The ANA positivity is poorly specific, and in the suspect of SARDs it is necessary to require further testing for anti-ENA (extractable nuclear antigen) specificities. With this term we refer to a group of autoAgs originally identified as antibody targets in people with SARDs that are commonly composed of non-histone ribonucleoproteins, called by the name of the donor who provided the prototype serum (i.e. Sm for Smith), or the name of the disease in which the antibodies were found (i.e. SS-A and SS-B for SjS antigen A and B; Scl-70 for the first identification in a SSc patient) [19]. In the suspect of SARDs, routine laboratory testing requires both ANA with titer and anti-ENA; for example, when we suspect SLE in a patient we could receive a report of homogeneous ANA pattern at high titer which is consistent with positivity to anti-dsDNA antibodies, and ENA may be positive for anti-Sm/RNP antibodies that are very specific for SLE and are also included in the 1982 ACR SLE classification criteria [20].
As mentioned, autoAbs are central to the diagnosis of SARDs and are included in the diagnostic and classification criteria of several diseases (**Figure 1, panel B**). In particular, ANA, anti-dsDNA and anti-Sm/RNP are part of the SLE diagnostic criteria established in 1982 [20]; anti-Ro/SSA and anti-La/SSB antibodies are included in the SjS classification criteria defined by Vitali et al [21]; for the diagnosis of SSc, the identification of three autoAbs (anti-centromere, anti-topo I/Scl70, anti-RNA polymerase III) is included in the last revision of SSc diagnostic criteria published in 2015 [22]; the presence of anti-Jo-1 antibodies identifies patients with the so-called anti-synthetase syndrome [23].

*1.3- Immunoprecipitation.*

In the last decade, several new methods have been developed to increase the number of tests to be performed in routine autoimmunity laboratories and to use quantitative automated high-volume solid phase methods instead of standard manual methods. These new assays have been first tested in dedicated specialized laboratories but now they are used in high-throughput routine laboratories and this has raised the problem of quality control mainly for reproducibility, sensitivity, specificity and clinical interpretation [24-26]. These problems have become so important that in 2010 a task force organized by the American College of Rheumatology (ACR) has developed a set of recommendations for ANA testing [16], stating that IIF remains the gold-standard for ANA testing and that in-house assays for autoAbs testing should be standardized according to national or international standards (i.e. CDC).

As for immunoprecipitation (IP), this is the method preferred for the identification of autoAbs in SARDs [2, 27] as it allows the first identification of many autoAbs that are then used in routine clinical practice though the development of commercial assays, as for anti-RNApol III antibodies in SSc patients. These were identified and tested by IP for decades until the development of a specific anti-RNApol III ELISA that is currently used in routine autoimmunity laboratories [19]. IP has the advantage of showing not only known but also unknown autoAbs in a single assay, and to allow the further identification of the bands of the target autoAgs using mass spectrometric analysis of purified proteins. These positive aspects of IP are associated to some disadvantages for the use of IP, mainly represented by the fact that it requires dedicated personnel, it is not automated, it requires experience in the set up and interpretation of results, and it needs dedicated equipment and radioactivity for protein-IP. For these limitations, IP is used only in a few research laboratories worldwide, and the need for quick and high-throughput methods led us to patent an alternative method for the identification of two autoAbs detected in SSc patients, called anti-Th/To and -U3/fibrillarin, through the combination of IP samples preparation followed by RT-qPCR [28]. This method avoids the need to run urea-PAGE gels that are very delicate and that lead to qualitative and not quantitative results in the detection of RNA bands. However, the use of methods alternative to

IP for autoAb analysis (such as ELISA and immunoblotting) still needs validation and it is still matter of debate in the international rheumatology community.

*1.4- Autoantibodies identified in SARDs by IP*.

The importance of autoAbs has been progressively highlighted by their inclusion in several sets of classification and diagnostic criteria, starting from the first sets of criteria in the 1970s-1980s for SLE and PM [20, 29] and followed by the most recent criteria for SSc in which autoAbs were considered as important as specific clinical features and capillaroscopy alterations in the classification of patients [22]. **Table 1** shows the main serum autoAbs that can be identified by protein-and RNA-IP in SSc, PM and DM patients, with a description of the target antigen and its features recognized by protein-IP and/or RNA-IP. For SSc, the identification of ACA is still based on IIF, even if it is possible to detect specific centromere proteins (CENP A-F) by other techniques such as line blot, immunoblot and ELISA [27]. No ACA specific band can be detected by IP, even if we show the presence of a new pattern associated to ACA in our Results section. Other SSc autoAbs can be identified by IP based on their target antigen: topo I is present as a strong 110kD band with a smear toward high molecular weight, presumable due to phosphorylation [30]; RNA polymerase antibodies are identified through a set of several bands as described in **Table 1** [31]; Th/To are clearly visible by RNA-IP through the reactivity of the two RNA bands called 7-2 and 8-2 RNAs [32, 33]; similarly, U3/ fibrillarin antibodies can be identified by the U3RNA band at RNA-IP as first described in 1985 [34] and –PM/Scl that characterize the overlap between PM and SSc features are detected by protein-IP [35, 36]. As for PM patients, we can identify several anti-synthetase antibodies by protein- and RNA-IP [37] (**Table 1**), and in the last decade several new autoAbs have been detected in PM patients thanks to these techniques: this is the case of the less common anti-synthetase antibodies such as anti-PL-12, PL-7, EJ, and OJ that seem to be associated with peculiar clinical manifestations within the classical anti-synthetase syndrome [38-43]; and also the anti-SRP antibodies usually associated to a form of necrotizing and not inflammatory myopathy [44]. Another field of active research of autoAbs is represented by DM, in which the only known

autoAb has been anti-Mi-2 for decades [45], but in the last decade several other autoAbs such as anti-MJ/NXP-2 [46], -MDA5 [47], -TIF1β [48], -TIF1γ/α [49] were identified and their clinical correlations have been increasingly studied in different ethnic groups [37].

Beside the mentioned autoAbs listed in **Table 1**, several other autoAbs can be identified but they are not considered specific for particular SARDs. This is the case of anti-Ro/SSA (Ro60) and -La/SSB that can be identified in several conditions, first of all SjS but also SLE, SSc and PM/DM, or anti-Ro52 and Ago2/Su antibodies that have been identified in several conditions without a clear clinical significance [50].

## 2. REPRESENTATIVE DISEASES INCLUDED IN THE PRESENT WORK.

### *2.1- Systemic Sclerosis (SSc).*

SSc is an autoimmune disease of unknown origin characterized by microvascular damage and progressive fibrosis of skin that in severe cases can affect internal organs such as heart, lungs, and kidneys. As most autoimmune diseases, it is more common in female patients (female:male 4:1) with age of onset at 30-60 years. In Italy, it is considered a rare disease due to its low prevalence, which is estimated to be 1/6.500 adults in the general population, with differences linked to ethnicity and geographic areas [51]. One of the typical symptoms at SSc onset is the Raynaud's phenomenon, expression of severe peripheral vaso-constriction, but the clinical aspects of SSc can be very heterogeneous and they can develop sometimes very quickly and aggressively, and sometimes more slowly and over a period of decades [52]. The etiology and the pathogenesis of SSc are unknown, but two processes, fibrosis and microvascular occlusion, characterize the pathological findings seen in all involved organs in SSc patients [53]. Several mechanisms are proposed in the pathogenesis of SSc disease, such as (i) activation of the adaptive and innate immune system and activation of endothelial cells; (ii) release of cytokines (i.e. TGF-β, PDGF, IL-4) from platelets, macrophages, and T-cells; (iii) cytokine activation of fibroblasts to increase extracellular matrix production; (iv) altered expression of VEGF [54, 55]. Concerning this aspect, we studied levels of serum VEGF concentration in our SSc patients, and we could see that this

molecule is variably expressed in SSc patients with pulmonary interstitial involvement [56] and *unpublished data*] and it could be considered as a biomarker of this specific clinical feature in SSc. The two main forms in which SSc can develop in patients are called diffuse or limited according to the extent of skin fibrosis as established by LeRoy in 1988 [57]. Each of these forms is associated with the presence of a specific autoAb profile, and the great importance of autoAbs in SSc is demonstrated by their inclusion in the recent diagnostic criteria developed by ACR/EULAR in 2013 [22]. In fact, the occurrence of ANA is present in virtually all SSc patients [58] and they are very characteristic [27, 59, 60] in SSc patients for the following reasons:

1- they are specific for SSc, as they are not detected in other autoimmune diseases or immune-mediated mechanisms;

2- they are positive since the onset of SSc and they don't change during the disease course;

3- they are unique and mutually exclusive [61], because the detection of multiple positivity in the same SSc patient is very rare;

4- they characterize clinical subsets of SSc (diffuse vs limited), and also particular clinical manifestations in SSc, for example anti-RNAPIII antibodies are usually identified in diffuse SSc with high risk of scleroderma renal crisis [62, 63].

The most frequently identified autoAbs in SSc are ACA (30%), anti-topo I (30%), and -RNA pol III (4-20%), and in fact these three specificities are the only ones included in the 2013 ACR/EULAR criteria for SSc diagnosis [22]. However, several other autoAbs can be identified in SSc patients and they can have an important role for their clinical and prognostic value, as for anti-U3/fibrillarin and anti-Th/To antibodies [1]. One of the main limitations in the study of autoAbs in SARDs such as SSc is the lack of routine methods of testing [27, 64] that could be used worldwide. In fact, protein- and RNA-IP still represent the gold standard for the analysis of antigenic components recognized by sera of SSc patients, and for this reason we applied these techniques to our SSc patients without known autoantibody specificity (ACA and anti-topo I negative) despite ANA positive pattern and high titer.

*2.2- Poly/Dermatomyositis (PM/DM).*

PM/DM are two conditions within the spectrum of Idiopathic Inflammatory Myopathy and they are CTDs characterized by muscle inflammation (myositis) and in the case of DM also skin inflammation with unknown etiology and pathogenesis. Several hypotheses have been proposed and discussed as triggers of PM/DM, and among these we have genetic predisposition, infections (i.e. CMV, EBV) and cancer in PM/DM paraneoplastic forms [65], but no certain factor has been identified so far [66] *and Ceribelli et al, Clin Rev Allergy Immunol 2015, in press*]. Both PM and DM are rare diseases because of their low prevalence and incidence in the general population (annual incidence of 5-10 cases/million people; prevalence 6-7 cases/100.000 people), and they affect female more than male (ratio 2.5:1) with bimodal distribution: some cases are defined juvenile because the onset is typically at 5-15 years of age, while other cases are diagnosed in adult people aged 30-50 years [67, 68]. Myositis is the main symptom of PM/DM, represented by muscle inflammation that can be diagnosed based on histological analysis of muscle biopsy, which can differentiate PM and DM based on the location of the inflammatory infiltrate, usually perivascular in DM while it surrounds muscle fibers in PM [65]. Myositis is described by patients as symmetrical proximal muscular weakness, and it can affect muscle fibers at different sites causing severe symptoms such as dysphagia and respiratory-muscle weakness. If not treated, muscle inflammation leads to fibrosis and muscle atrophy which are responsible for severe and irreversible disability [65, 69]. Inflammation in PM/DM can be systemic and it may be associated with other symptoms such as lung inflammation (interstitial lung disease), skin disease, arthritis and Raynaud's phenomenon, and all these aspects worsen the PM/DM patients' prognosis and therapeutic benefits of immunosuppressive medications [69]. The first diagnostic criteria for PM/DM were published in 1975 by Bohan and Peter [29, 70] to define PM and DM mainly based on clinical, instrumental and histopathological criteria. AutoAbs were not present in this first set of criteria, and they were included only later, in the diagnostic criteria proposed by Targoff in 1997 [23]. In fact it had become increasingly clear that autoAbs are very important in PM/DM patients

because they are positive in about 50-80% of cases and they have clinical associations that help the clinician in patients' diagnosis and classification [71]. This is particularly true for anti-Jo-1 (histidyl-tRNA synthetase) antibodies that were included in this set of new classification criteria defined by Targoff et al, and they are present in 15-20% of patients affected by PM with a specific syndrome called anti-synthetase syndrome characterized by a set of symptoms such as myositis, interstitial lung disease, polyarthritis, mechanic's hands, fever and Raynaud's phenomenon [72]. This is a clear example of how antibodies can define specific clinical features thus having a predictive and prognostic value [72]. However, many PM/DM patients (up to 50%) still do not have known autoAbs despite the presence of high-titer ANA by IIF. Clinical research is going on to identify target autoAg in PM/DM patients, and the increased number of cases collected worldwide is of great importance also to characterize the clinical phenotype associated to specific autoAbs. For this reason, since 2015 we became part of the Euromyositis Network (https://euromyositis.eu) which represents an International collaboration research and treatment database for myositis specialists.

*2.3- Psoriatic Arthritis (PsA).*

Arthritidies can be defined as seropositive when the characteristic RF and anti-CCP antibodies are present, or they are called seronegative when these antibodies are negative and the diagnosis can be made only at the moment of clinical onset of arthritis [73-75]. Rheumatoid Arthritis (RA) is the prototype of seropositive arthritis, while in the group of seronegative arthritis we include psoriatic arthritis (PsA, associated to skin or nail psoriasis), reactive arthritis (secondary to genitourinary or bowel infections), spondyloarthritis (affecting joints and spine), and enteropathic arthritis (associated to inflammatory bowel diseases such as Crohn's disease and ulcerative colitis) as shown in **Figure 1 panel A** [6, 76]. Differently from RA, PsA is characterized by inflammation not only of joints but also of peri-articular structures, leading to manifestations such as arthritis, enthesitis and dactilytis. No present parameter can be used for the diagnosis of PsA, and the only method of evaluating risk of spondylitis is the positivity to HLA-B27 gene and/or the increase of inflammation

markers such as ESR or CRP [77, 78]. Skin psoriasis (PsO) is a common condition affecting 2-4% of the general population, but only 25-40% of PsO patients can develop PsA and skin disease may develop after the onset of the joint inflammation, so this factor does not always help in the diagnosis of PsA, and a strong effort has been done recently in the search for a new biomarker of PsA [79]. Among the new biomarkers, the interleukin IL23/Th17 pathway has been promising mainly for pathogenic and therapeutic approaches [80], while the analysis of PsA patients through several approaches such as multiplex panels, proteomics, epigenetics and genetic alterations different from HLA-B27, have been proposed to help in the identification of a new PsA biomarker for early diagnosis and follow-up, but also for the response prediction to biologic therapies [81-84]. The etiology of PsA is unknown, but recent reports have supported presence of serum autoAbs with cross-reactivity for autoAg shared by skin and joints [85]. These reports show a possible autoimmune origin of PsA, and a role of skin antigens in the activation of innate and adaptive immunity in PsA that may be represented by a molecule called LL37 that has been described as the autoAg inducing the T-cell response in the skin of PsO patients. LL37 is a cleavage product of one of the major human anti-microbial peptides, cathelicidin [86], and it seems to have DNA- and RNA-sensing capacity and immunomodulatory properties (i.e. chemotactic function; pro-angiogenic action) important in skin immune defense and disease. LL37 basal production in the skin is physiologically low, while it is overexpressed in PsO plaques and in the serum of patients with PsO [87] and it is the only form of cathelicidin found in PsO skin [88]. In PsO plaques, extracellular DNA and RNA released from dying cells together with increased LL37 levels constitute a pro-inflammatory stimulus initiating and amplifying cutaneous inflammation via dendritic cell activation [89]. However, LL37 also plays an anti-inflammatory role by targeting another key player in PsO pathogenesis, IL-1beta, via the AIM2 inflammasome [90]. In patients with RA and in a murine model of arthritis an increased expression of LL37 in the synovium and serum anti-LL37 autoantibodies have been observed [91], thus suggesting a role of B cell-mediated LL37 response in arthritis pathogenesis. LL37 has been identified in the skin microbiota also in

newborn infants, and it is possible that due to environmental factors (i.e. keratinocyte apoptosis after UV exposure) and/or genetic predisposition it could trigger an autoimmune response against self-antigens that are responsible for PsO and in predisposing people also to PsA [92]. Moreover, LL37 may be able to alter the immune system of the host through the interaction with APCs and then activation of B and T cells against self-antigens for mechanisms of molecular mimicry. In fact, LL37 is not only present in the skin but circulating LL37 T-cells have been identified in PsO patients [93] and they may be responsible for the activation of the innate and immune response also leading to PsA.

Based on these recent reports we decided to study our PsA patients in two different directions: 1) screening of our PsA patients by protein- and RNA-IP in order to evaluate the possible presence of autoAbs to be used as serum biomarkers for the diagnosis, clinical association and therapeutic choices in PsA patients; 2) analysis of LL37 concentration in the serum and in the synovial fluid of PsA patients, to evaluate the presence of this component as marker of disease or inflammation activity.

*2.4- Behçet Disease (BD).*

Over the last 25 years we witnessed strenuous efforts to set classification and diagnostic criteria adequate for vasculitidies, but no clear result has been achieved [94-96]. With a few exceptions represented by Takayasu arteritis [97], polyarteritis nodosa [98] and Behçet Disease (BD) [99], all other vasculitidies have no recognized classification criteria. In 1994, a set of classification criteria was defined by the Chapel Hill group in which the size of the affected vessels was used to discriminate among the different vasculitis [100], and these criteria were further revised in 2012 [101] also to add the difference between primary and secondary forms, for examples those associated to hepatitis B or C. No biomarker has been identified to help in the distinction of these several vasculitis, so the diagnosis still is performed on a clinical, instrumental or histological setting leading to different therapeutic approaches [102]. The only autoAb identified so far in a few vasculitis is represented by serum ANCA (anti-neutrophil cytoplasm antibodies) discovered in the

mid-eighties, but no further progress has been made in this field of research for vasculitis. ANCA can be classified as P (perinuclear) or C (cytoplasmic) according to the pattern visible by indirect immunofluorescence staining, but this requires confirmation by solid phase assays (i.e. ELISA) for the antigen recognized, that is proteinase 3 (PR3) by c-ANCA and myeloperoxidase (MPO) by p-ANCA [103-105]. BD belongs to the family of vasculitis because it is a chronic vascular inflammatory disease characterized by symptoms affecting internal organs and usually characterized by three main symptoms: oral and genital aphtous lesions and ocular manifestations such as uveitis [106]. BD can be characterized less frequently by additional symptoms such as arthritis, renal and gastrointestinal disease, and skin lesions revealed by the pathergy test [106]. BD is a rare disease as the estimated prevalence in Europe of 1,5-15,9 cases every 100.000 inhabitants in Europe, with highest number of BD patients in Turkey (20-420 cases every 100.000 inhabitants) [107, 108], and it mainly affects male patients contrary to what observed in most autoimmune diseases. The etiology and the pathogenic mechanisms are not known, and controversy is still present for what concerns its inflammatory or autoimmune nature [109]. Previous reports have shown the presence of autoantibodies in BD patients, such as ANCA [110], ASCA (antibodies against saccharomyces cerevisiae) [111], and antibodies against phosphatidylserine and ribosomal phosphoproteins [112], but no specific signature autoAb has been identified so far, and for these reasons we performed protein- and RNA-IP also on a set of patients affected by BD.

## UNMET NEEDS.

**Unmet need# 1.** There are no serum biomarkers for a reproducible, non invasive, early diagnosis of systemic autoimmune rheumatic diseases and previous reports clearly showed that a 2-year treatment delay is associated with irreversible joint and organ damage. In particular rare conditions such as SSc, PM/DM, seronegative arthritis, and vasculitis are characterized by a plethora of poorly-prevalent serum autoAbs, often with clinical implications in the small minority of positive patients. We foresee that a more complete panel of autoAbs in larger cohorts of patients would allow a better definition and ultimately impact the management of these cases.

**Unmet need# 2**. Uncommon presentations and clinical phenotypes are challenging possibilities in the management of rare SARDs. The identification of new serological markers such as autoAbs in SARDs could increase the chance for early diagnosis and treatment also for patients who have less common and specific disease features.

## GENERAL AIMS

**Aim# 1.** To set up protein and RNA-IP in our laboratory for routine testing of samples from patients affected by SARDs.

**Aim# 2.** To identify known, rare and new autoantibodies in patients affected by SSc, PM/DM, PsA and BD, through the analysis of immunoprecipitated bands.

## PATIENTS AND METHODS

### *Patients*

Sera from 188 consecutive patients affected by SARDs were collected in our Rheumatology outpatient clinic in 2013-2015 and stored in our laboratory of Autoimmunity and Metabolism in Humanitas Research Hospital (Rozzano, Milan, Italy). Each serum sample was tested once by protein-IP and once by RNA-IP, and positive samples were retested for confirmation. Inclusion criteria for sera collection were: (i) diagnosis of SSc with positive ANA (with a nucleolar, speckled, or homogeneous pattern, different from centromere -ACA), and negative ENA for serum anti-topo I and –RNA pol III; (ii) diagnosis of undifferentiated connective tissue disease (UCTD)/pre-SSc, a condition in which the systemic autoimmune disease does not fulfill the accepted classification criteria for a specific CTD [113] and in the case of pre-SSc patients have only few disease features (i.e. Raynaud's phenomenon, nailfold capillaroscopy changes) with ANA positive antibodies but no other clinical feature suggestive of SSc [114] ; (iii) diagnosis of PM/DM with unknown ENA despite ANA positivity at routine clinical testing; (iv) diagnosis of PsA following the CASPAR criteria [6]. Through these inclusion criteria we were able to collect 63 SSc sera, 22 sera from patients affected by Idiopathic Inflammatory Myopathies such as PM/DM and anti-synthetase syndrome, and 58 sera from patients affected by PsA, to perform protein- and RNA-IP (**Figure 2**). An additional set of sera from 45 patients affected by BD was collected by our collaborator Dr. Luca Cantarini (U.O.C. Reumatologia, Dipartimento di Scienze Mediche, Chirurgiche e Neuroscienze; Azienda Ospedaliera Universitaria Senese; Ospedale Santa Maria alle Scotte; Siena, Italy) and tested by protein and RNA-IP (**Figure 2**).

For the analysis of concentration of LL37 in PsA patients we tested serum and synovial fluid samples obtained in the same visit with venipuncture and knee arthocentesis, respectively, for three consecutive patients with active PsA evaluated in our outpatient clinic in 2014.

The study has been conducted in accordance with the Declaration of Helsinki and its subsequent modifications. All patients provided written informed consent and the protocol was reviewed and approved by the local ethical committee.

***Methods***

***SERUM AND SYNOVIAL FLUID.***

Patients' serum (and plasma) is obtained from whole blood through centrifugation at 2000g for 15 minutes, and then stored in -20°C freezer until use. Synovial fluid is centrifuged at 980g for 10 minutes and divided in aliquots stored in -80°C freezer until use.

***REFERENCE SERA FOR IP (POSITIVE CONTROLS).***

As reference sera to validate protein- and RNA-IP, we used ANA reference standards obtained from the Centers for Disease Control and Prevention (CDC, Atlanta, USA) that are available for clinical or commercial laboratories as quality controls, through the Autoantibody Standardization Committee (www.autoab.org) and the collaboration with the administrator of this website, Dr. E.K.L. Chan at the University of Florida (Gainesville, USA). We received 19 reference human sera for our experiments: homogeneous/RIM pattern, La/SSB, speckled pattern (U1-RNP,SSB, SSA), U1 RNP, Sm nuclear antigen, nucleolar pattern (U3RNP/fibrillarin), Ro/SSA, centromere pattern (ACA), topoI/Scl70, Jo-1, PM/Scl (PM-1), rRNP/Ribosomal P, MPO-ANCA, PR3-ANCA, anti-CCP, anti-cardiolipin (epitope beta2 GPI)EY2C9, and anti-cardiolipin (epitope beta2 GPI) HCAL. They were lyophilized and resuspended with 500µl of deionized water as recommended, just before use, and then stored at -20°C.

***PROTEIN-IMMUNOPRECIPITATION (IP).***

The following protocol has been modified from [115]:

Cell culture: radiolabeled cell extract is prepared using K562 cells (human erythroleukemia cell line). Cells are thawed 2-3 days before use and grown in RPMI-1640 with 10% FBS, 100x L-glutamine, 100x Pen-Strep, 100x HEPES in a 5% CO2 atmosphere, at 37˚C.

Radiolabeling: K562 cells are collected by centrifuge (300 X g for 5minutes in a tabletop centrifuge), in a concentration of $10^8$ cells cultured in 45ml of complete methionine-cysteine free $^{35}$S-labeling culture medium in a 250ml flask. A quantity of 4.2mCi of $^{35}$S-methionine/cysteine is added and cells are incubated for 12-14hours at 37 ˚C in a 5% CO2 atmosphere. Cells incorporate $^{35}$S-methionine/cysteine for protein synthesis during growth and proliferation. After 12-14hours radiolabeling, PBS is added to the culture and cells are divided into 15ml tubes, centrifuged (300 X g, 5min), supernatant is aspirated, and cell pellet is used fresh or frozen at -80˚C until use.

Protein A Sepharose (PAS) beads: PAS are prepared as solution 50% (v/v) with $dH_2O$, then a 1/100 amount of 2M tris-HCl pH7.5 is added to a final concentration of 20mM and 10% NaN3 to a final concentration of 0.1%. PAS beads can be stored at 4 ˚C in this buffer, until used.

Preparation of PAS beads with purified antibodies: serum (or plasma) stored frozen or at 4˚C can be used, usually 8 µl added to 40 µl of 50% (v/v) PAS beads, to allow IgG to bind to PAS immediately; 500µl of 0.5M NaCl NET/MP-40 buffer are then added and the rack is wrapped using plastic film and rotates for 1hour - overnight at 4˚C.

Preparation of radiolabeled cell extract: when the PAS beads with purified antibodies are ready for IP, cell extract is prepared. For a standard reaction, radiolabeled cell extract from ~2 x $10^6$ cells/sample is used; 2 ml of 0.5M NaCl NET/NP-40 are added to the frozen cell pellet (~2 x $10^7$ cells, final cell concentration $10^7$/ml), then 1:100 PMSF (Phenylmethylsulfonyl fluoride; 50mM in absolute ethanol) and aprotinin (from bovine lung aseptically filled solution in 0.9% NaCl and 0.9% benzyl alcohol) are added. Because a sonicator was not available for use with radioactive $^{35}$S-K562 cells, we tested two alternative methods for antigen extraction: (i) vortexing for 60 seconds for three times, leaving the pellet on ice between the cycles to avoid heating; (ii) 5 cycles of freezing in dry ice and thawing in 37°C waterbath for 1 minute at each step (**Figure 3)**. As shown in **Figure 3,** results were comparable among these methods, and we decided to use vortexing cycles in our protocol instead of sonication. After vortexing, cell lysate is transferred to microcentrifuge tubes and centrifuged at 9000 X g for 30 minutes at 4˚C. The supernatant is carefully collected to avoid

disturbing the small pellet and ~160 µl of cell extract/sample is a standard quantity used in this condition to run samples twice in acrylamide gels for screening purpose.

Incubation with cell lysate: the antibody-coated PAS beads are collected by microcentrifuging for 8 seconds, the supernatant is aspirated and two washes with 1ml of 0.5M NaCl NET/NP-40 are performed. To each tube with PAS beads, 160 µl of cell extract is added per sample. Tubes are placed on a plastic rack, wrapped with plastic film and rotate end over end for 1hour at 4 ˚C.

Washing beads: beads are washed 4 times as follows: microcentrifuge for 8 seconds, aspirate supernatant, add 1ml of 0.5M NaCl NET/NP-40, wash inside of the cap by inverting once, microcentrifuge for 8 sec. Then one wash is performed with the same steps but with 0.15M NaCl NET/NP-40 buffer. After the washes, 50µl of sample buffer (1.5ml 2M TRIS pH 6.8, 0.6ml 1% bromphenol blue, 3ml 2-mercaptoethanol, 4.8ml 25%SDS, 6ml glycerol, 4.1ml $dH_20$) are added and samples are frozen at -80 ˚C or -20 ˚C until use. Samples are boiled before running gels, for 3 minutes, and samples are then centrifuged for 8 seconds. After this step, they are ready to be used and usually 20-25µl of sample are used in each lane for the SDS-PAGE gel.

SDS-PAGE gels: 8% or 12.5% SDS-PAGE gels are run (running buffer: 1x Tris glycine, 90mM Tris, 90mM boric acid, 2mM Na2EDTA) to fractionate according to the molecular weight: the smallest proteins that can be fractionated on 8% gels are ~25kD, while proteins of >100kD are not separated well on 12.5% gels. When running large or medium size gels, it takes 3-5hours at a constant voltage of 100-130V for the tracking dye front to reach to the bottom of the gels. When gels are ready, the stacking gel is cut off (leave 1-2 mm of stacking gels) and gels are stained for 30-60 minutes with Coomassie blue solution (30% methanol, 10% gracial acetic acid, 0,1% Coomassie brilliant blur R250, 60% dH20). After this step, gels are destained in the destaining solution (35% methanol, 10% gracial acetic acid, 55%dH20) overnight.

Fluorography: after staining and destaining, fluorography is performed as follows:

1. DMSO (dimethyl sulfoxide) is added twice to eliminate water residues from the gel; for each cycle, gels are left in DMSO for 30 min on a shaker;

2. PPO (2.5-diphenyloxazole) is added after discarding DMSO to enhance weak signals from $^{35}S$; it is prepared as 20% (weight/volume) PPO in DMSO and the gel with PPO is left on the shaker for 1hour.

3. After these steps with DMSO and PPO, gels are washed several times to remove DMSO and make PPO precipitate in the gels. At this point, clear gels turn into white and opaque upon washing with water. Removing DMSO is also critical to make thinner non-sticky gels after drying. The washing step is repeated for at least 30 minutes. At this point, blue signals corresponding to immunoglobulin light and heavy chains, and the molecular weight marker are clearly visible.

4. When gels are ready for drying, glycerol is added to a concentration of ~3%.The gel is placed on a piece of filter paper and set on the gel dryer at 60 ˚C for 3 hours.

Autoradiography: after the drying step, in the darkroom the gel is placed in a film cassette in direct contact with X-ray (GE Healthcare) film. The half-life of $^{35}S$ is 89 days, and usually, the signal can be detected after 3 days of exposure, but if the radioactivity is weak, it is necessary to expose gels longer. The cassette containing exposed films is left for 3-5 days at -70 ˚C before developing. Detection of the radioactive signal was performed with developing reagents (fixer and developer, Kodak) for a variable time (1-15 minutes), depending on the intensity of the radioactive signal.

Protein-IP interpretation: Each film is labeled by sera code, positions of molecular weight marker, date of development, number of exposure days, and percentage of the SDS-PAGE gel. As a basic rule in interpretation of IP, observation of any protein bands that are present in a particular lane but not in other lanes means that they are specifically recognized by antibodies in the serum. Interpretation of specificity of autoAbs by IP is primarily based on the mobility of immunoprecipitated proteins by the sample, ideally by comparing with the size and the pattern of proteins immunoprecipitated by reference sera. Reference sera (positive controls) and negative controls (normal human serum) are run in every gel.

***RNA-IMMUNOPRECIPITATION (IP).***

The following protocol has been modified from [115]: small RNA components of autoAg recognized by sera from SARDs patients can be identified based on IP analysis of RNAs similar to the above described immunoprecipitation procedure for proteins using radiolabeled K562 cells. The main differences in the protein- and –RNA-IP protocols are shown in **Table 2**. Due to concerns for strong radiation produced by $^{32}$P-orthophosphate labeled cells that was used for RNA-IP, silver staining analysis of RNAs is now the non-radioactive procedure most used worldwide, and it is the technique that we set up in our lab. Steps for preparing PAS beads stock and incubation with sera are the same as the method for protein-IP. Beads after incubation with sera can be stored in at 4°C for several days in NET/NP-40 buffer that must be aspirated before adding cell extract.

Cell lysate: Unlabeled K562 cells (either frozen pellet or fresh cells) are used at 5 x$10^6$ cells/sample because this concentration of cells is considered enough to detect RNA components for all autoAb that recognize RNA-protein complex. For the RNA-IP protocol we were able to use the sonicator for 45 seconds (twice) at duty cycle 30%, output control 3 (Branson, Sonifier) because K562 cells are not radiolabeled. K562 cell lysate is then transferred to microfuge tubes, centrifuged for 30 min at 9000 Xg at 4°C. PAS beads incubated with sera are washed while clearing the cell lysate. The supernatant is carefully collected avoiding cell pellet and cell extract is added on beads, usually 200 microliter/sample but this volume can be adjusted depending on experiments. Samples are put on a plastic rack, wrapped with a plastic film and rotated at 4°C for 1hour.

Preparation of total RNA sample as a standard: 20 microliter (extract from ~5x$10^5$ cells) of cell lysate are put in an Eppendorf tube, and 400 microliter of 0.15M NET/NP-40IGEPAL, 16 microliter of 25% SDS, 40 microliter of 3M Na acetate pH 5.2 are added. For RNA extraction, 400 microliter of phenol/chloroform/isoamyl alcohol (25: 24: 1, pH 5.2 for RNA extraction; Fisher) are added. Each Eppendorf tube is then vortexed vigorously for 1 minute, and spinned for 1 minute at room temperature at 9000 Xg. Supernatant is transferred to a clean new tube avoiding the protein layer to avoid contamination, in a volume of 300 microliters, and 900 microliter of 100% (200

proof) ethanol are added, then mixed well by inverting tubes. Total RNA sample is ready after leaving the tubes at -80°C overnight and centrifuge at 12000 rpm for 15 minutes.

Washing beads: this step is performed as described for protein-IP (see protocol above).

Extraction of RNAs from the immunoprecipitates: as for total RNA preparation as standard, 400 microliter of 0.15M NaCl NET/NP-40, 16 microliter of 25% SDS, and 40 microliter of 3M Na acetate pH 5.2 are added to each sample after incubation with K562 cell lysate and the washing steps. For RNA extraction, 400 microliter of phenol/chloroform/isoamyl alcohol (25: 24: 1) are added and each sample is first vortexed vigorously for 1 minute, then spinned for 1 minute (9,000 Xg at room temperature). A volume of 300 microliters is harvested from the top layer, avoiding the protein layer, and 900 microliter of 100% (200 proof) ethanol are added, then samples are mixed gently by inverting tubes. Tubes are left at -80°C overnight.

Preparation of RNA samples for urea-PAGE: the following day, RNA samples are microcentrifuged at 9,000 Xg for 15 minutes in a cold room, supernatant can be discarded and aspirated using a thin Pasteur pipette. Tubes are left under biological safety hood to dry.

Preparation of urea-PAGE gel: RNA samples are run in a 12% urea-PAGE gel, prepared as follows: 21 g of urea, 20 ml of 30% acrylamide/bis, 5 ml of 10 X TBE, 300 microliters of ammonium persulfate (10% APS in milliQ water), and 30 microliter of TEMED in final volume of 50ml adjusted with milliQ $H_2O$.

Urea-PAGE: when the gel is ready, a pre-run at 400 volt constant voltage is started trying to keep the gel temperature between 55 - 60°C (running buffer 1xTBE). A volume of 50 microliter of urea-PAGE sample buffer is added to each sample tube and vortexed to dissolve RNA. After the pre-run, samples are loaded in the UREA-PAGE gel (20 microliter/lane) after washing quickly each well using Hamilton microsyringe. In fact, high concentration of urea keeps coming out from the gel into the wells and may disturb samples to stay in the bottom of wells. The run is started at 400V and it continues for 1.5-2hours until the xylene cyanol dye (second dye in purple color) reaches the bottom of the gel (it may go out).

Silver staining of nucleic acids (Bio-Rad Silver Stain Plus): follow the manufacturer's instruction. After detecting signals gels were dried for 2 hours at 60°C on a gel dryer and conserved.

RNA-IP interpretation: RNAs detected in RNA-IP samples become visible bands in the urea-PAGE gel and their migration allows the correct interpretation when compared to total RNA as standard and to positive reference sera. For example, the positivity of anti-Th/To antibodies is recognized by the immunoprecipitation of 8-2 and 7-2RNAs, while anti-synthetase antibodies recognize autoantigens that have tRNA as a component (i.e. $tRNA^{his}$ for anti-Jo-1).

**IP-WESTERN BLOT for anti-hnRNP antibodies in SSc patients.**

1- Crosslinking of IgG to PAS beads: the following protocol was used and modified from [116]: 50 µl of patients's sera and 500 µl of 0.5M NaCl NET/NP40 are added to 60µl of 50% (v/v) PAS beads and incubated overnight. The following day, PAS beads are washed twice with 1ml 0.5M NaCl NET/NP40. Each sample is then washed 3 times with 1ml of 0.2M triethanolamine (TEA) pH 8.4, then 1ml of 20mM DMP (dimethyl pimelimidate) in 0.2M TEA Ph 8.4 are added and samples rotate at room temperature for 2 hours. Samples are then washed with 0.2M TEA and blocked with 1ml of 50mM TRIS HCl pH 7.6, they rotate at room temperature for 1 hour and samples are then washed with washing buffer (0.5M NaCl NET/NP40) for IP.

2- IP-Western Blot: This is the protocol we followed, modified from [117]: cell extract from unlabeled K562 cells (~70million cells; sonication is performed with two 45-seconds cycles after adding 4ml 0.5M Nacl NET/NP40, 40µl PMSF and 40µl Aprotinin). While spinning the cell extract at 12.000RPM for 30 minutes in cold room, samples are washed once with 1ml 0.5M NaCl NET/NP40. The K562 cell supernatant is collected and divided in the samples (ex. 200µl/sample) that rotate for 1 hour in the cold room. After preparing 8% SDS-PAGE gel (stacking+resolving small gel, as in the protein-IP protocol), and after adding the running buffer (1 liter for 2 gels= 800ml $dH_20$+200ml 5X tris-glycine+ 4ml 25%SDS), samples are added (15 µl/well of samples and 7µl of molecular weight marker) and run at 100V for 10 minutes, then increased to 130V (total run

time of 1 hour). At the end of the run the gel is positioned in the transfer buffer (48mM TRIS, 39mM glycine, 20% methanol, pH 9.2) and the transfer is started for 90 minutes at 300A constant. After the transfer, the membrane is placed in Ponceau Red for a few minutes, then it is removed and the membrane is washed for a few times with $dH_20$ and blocking buffer (5% milk in PBST) is added overnight.

As primary Ab we used mouse monoclonal anti- hnRNPC1+C2 (Abcam; 1:1000 diluted in blocking buffer+PBS) and anti-hnRNP-L (Abcam; 1:2000 in blocking buffer+PBS), incubated for 3 hours. After primary Ab incubation, we washed the membrane 3 times with TBST, and then incubated each membrane with HRP goat anti-mouse IgG (Fisher Scientific; 1: 3000 diluted in blocking buffer+PBS) for 45 minutes. After incubation with the secondary Ab, we washed the membrane 3 times with TBST and then added the chemiluminescent reagent (Millipore) for 5 minutes. The signal was detected after exposing the membranes to the X-ray film (GE Healthcare) for 10 seconds, and the film is then developed by fixer and developer liquids (Kodak).

**ANTI-LL37 ELISA.**

LL37 concentration in patients' sera (40µl) and synovial fluids (40µl) was measured by ELISA (Bioassay Technology Laboratory) according to manufacturer's instructions. Optical density was measured by VERSAmax microplate reader and analyzed using SoftMax Pro program (Molecular Devices, Inc), then converted into units using a standard curve created by standards included in the ELISA.

**STATISTICAL ANALYSIS.**

The Student's t test, Mann-Whitney or Fisher's exact test were used for comparisons between groups. In the analysis of LL37 concentration, values were analyzed as units and the cut-off was established as mean ± 3 standard deviations (SD). Statistical significance was defined as $p \leq 0.05$. Statistical analyses were performed using Prism 6.0 for Windows (GraphPad Software Inc., La Jolla, CA).

## RESULTS

### 1- REFERENCE SERA.

To verify the correct performance of protein-IP, we tested reference sera known to immunoprecipitate specific antigens with RNA and protein components that are recognized based on their molecular weight, as shown in **Figure 4**. In particular, we tested anti-Ro/SSA, -La/SSB, -topo I positive samples and one normal human control (**panel A**). We were able to see the IP patterns of these autoAbs based on the evidence of Ro 60kD antigen, La 48kD antigen (associated to weak Ro60 positivity as commonly seen), and topoI 110kD antigen, while no specific band was detected by the normal human serum (NHS). Similarly, we tested for RNA-IP some of the CDC reference sera that are known to immunoprecipitate RNA bands, as shown in **Figure 4, panel B**. We were able to detect anti-Sm antibodies through the identification of U2-U1-U4-U5-U6 RNA bands, we identified the-U1 RNA band in the anti-U1RNP positive reference serum, and we observed the Y-RNAs that characterize anti-Ro/SSA antibodies in their nucleic acid component. These specificities were all run together with total RNA samples as standard. Negative samples have no band in RNA-IP (**Figure 4, panel B**).

These results demonstrate the adequate set up of protein- and RNA-IP to identify reference autoAbs through the identification of their known antigenic patterns and molecular weight.

### 2- SYSTEMIC SCLEROSIS (SSc).

We tested by protein-IP and RNA-IP the SSc sera collected in the years 2013-2015 in our outpatient clinic. These patients (n=63) were consecutively enrolled from a total of 130 SSc patients followed regularly in our outpatient clinic, and we initially excluded SSc patients already characterized by routine clinical testing as ACA (61 cases) and -topo I (21 cases) positive, because these autoAbs are considered to be mutually exclusive.

Through protein-IP and RNA-IP we were able to identify additional autoAbs in SSc patients, defined as rare autoAbs that are not routinely tested by autoimmunity laboratories worldwide

(**Tables 1 and 3**). These SSc sera were considered negative for ENA autoAbs until that moment despite ANA positive pattern also at high titer and clear manifestations of SSc disease. Thanks to the use of protein- and RNA-IP in our lab we were able to reduce the number of SSc sera with unknown antibody from 14 to 4 (**Figure 5, panel A**), thus giving important diagnostic and prognostic information to the clinician for better management of SSc patients. Moreover, thanks to the identification of uncommon autoAbs we can use these sera as internal standards for future reference as positive controls, instead of using CDC reference sera. In particular we were able to detect the following uncommon SSc autoAbs: anti-Th/To (n=2), -SRP (n=1), -Ago2/Su (n=1, associated with –topo I), and -U3RNP/fibrillarin (n=3) **(Figure 5, panel C**). One SSc serum showed positivity for anti-replication protein A (RPA) antibodies (**Figure 5, panel B**). This autoAb is a rare specificity recognized by the heterotrimer of subunits of 70/32/14kD, identified so far in patients affected by SLE and SjS, but not in SSc patients. The rarity of this autoAb is well reflected by the identification of 9 cases out of 1119 sera screened by protein-IP in a previous report, and only 5 of these anti-RPA (+) cases were affected by SARDs [118]. It is unclear whether anti-RPA antibodies are associated with a unique clinical and immunological subset, and further analyses in positive cases may help in this direction, but we can say it was positive in our patient with diffuse SSc, severe Raynaud's with digital ulcers, lung and esophageal involvement, who recently developed thyroid cancer.

An additional important aspect in the use of IP for screening purpose is that this technique can be used also to identify new autoAbs. In our cohort, we were able to identify two new patterns. First, we were able to see a new pattern characterized by a common set of several proteins of 140/40-25kD in 8 SSc cases (**Figure 5, panel D**). This pattern is significantly associated with ACA positivity (p=0.008), and these patients have severe Raynaud's with digital ulcers requiring IV prostacyclin (75%, 6/8 vs 7/54 ACA+, 13%; p=0.0006), with esophageal involvement in 4 cases. This IP pattern may correspond to the complex called hnRNP (heterogeneous nuclear ribonucleoprotein) which is composed of several proteins and RNAs involved in RNA processing

and splicing. Some of these proteins have molecular weight ranging between 32 and 45kD as the set of proteins we identified in our gel [119]. Several human autoAbs have been used to examine the components of the hnRNP complex, mainly from SLE, RA and MCTD patients, with the identification of predominant proteins in different diseases. For example, hnRNP-A2 (also called RA33) has been identified as an important autoAg in RA patients with a role in the disease pathogenesis [120]. This protein is the target of autoAbs called anti-RA33 that have been previously reported also in SLE and MCTD patients, in association with anti-Sm and -U1RNP antibodies [121].

To define the 140/40-25kD pattern identified in 8 SSc cases, we performed IP-WB using two anti-hnRNP antibodies selected by the molecular weight of the target antigen. In fact, the pattern we identified by protein-IP is consistent with the same pattern described for hnRNP proteins observed in Western Blot, but no previous study was conducted by IP using human sera from patients with SARDs [119]. We tested anti-hnRNP C1+C2 and anti-hnRNP L monoclonal antibodies in these 8 samples after preparation by crosslinking of IgG to PAS beads and protein-IP protocol as described above, followed by WB. Results show that 6/8 SSc anti-140/40-25kD samples are positive for hnRNP L (**Figure 5, panel E**), and this may represent a new target antigen of a subset of SSc patients with ACA positivity and severe vasculopathy. No result was obtained from the incubation of hnRNP C1+C2 monoclonal antibody, and further analysis with monoclonal antibodies directed against other hnRNP components is still necessary.

The second new pattern we identified is shown in **Figure 6** and is characterized by a set of four proteins with 75-50-40-34kd molecular weight, in 6 SSc cases of our cohort. This pattern looks consistent with the mitochondrial antigen complex targeted by serum AMA (anti-mitochondrial antibodies) that represent the hallmark of primary biliary cirrohosis (PBC) in SSc patients [122, 123], that we already previously identify in another set of PBC sera (*unpublished data*). PBC is a frequent comorbidity in SSc patients and it is commonly associated with serum ACA, and when we

retrospectively evaluated these 6 SSc cases with positive AMA pattern by IP we could identify ACA positivity at IIF and known AMA positivity by routine assays with PBC in two of them.

**3- POLY/DERMATOMYOSITIS (PM/DM).**

Myositis antibodies are divided into two main groups: the first group contains myositis-specific autoAbs that are identified almost exclusively in PM/DM patients, while the second one is for myositis-associated antibodies that can be present also in patients affected by other SARDs, as for anti-Ro/SSA antibodies that are common not only in PM/DM but also in SjS and SLE [72]. We tested by protein-IP and RNA-IP 22 sera collected from patients affected by PM (N=12, one of them affected by anti-synthetase syndrome) and DM (n=10) collected in the years 2013-2015 in our outpatient clinic. These patients were selected from a total of 38 PM/DM patients followed regularly in our outpatient clinic, based on: i) ANA positivity at high titer but no known anti-ENA specificity; ii) negativity for anti-Jo-1 antibodies that is the only specificity currently tested in routine autoimmunity laboratories. Through protein- and RNA-IP we were able to identify rare autoAbs shown in **Table 4** and **Figure 7 (panel A and B)**, and in particular: anti-Mi-2 (n=2), -TIF1γ/α (n=2), -140kD (n=1), -Ro60 (n=5), -Jo-I (n=3), -EJ (n=1), -PL-12 (n=1) and -SRP (n=1) antibodies. The two patients with anti-Mi-2 antibodies have typical DM with characteristic skin manifestations and mild myositis, and they had good response to immunosuppressive therapy as reported in literature [72]. In patients positive for anti-TIF1γ/α antibodies, in one case this autoAb was identified in a young female patient with history of cancer (Non-Hodgkin's Lymphoma) 7 years before the onset of DM, while the other case developed in a 65-year old man with typical features of DM. This autoAb is known to be associated with a paraneoplastic forms of PM/DM, and it has a bad prognostic value when identified in adult patients, while in juvenile forms it is not associated with paraneoplastic myositis, but our female DM anti- TIF1γ/α (+) patient represents an exception to these reports [124]. One band visible at 140kD molecular weight was identified in a patient affected by DM, and this is suggestive for anti-MJ or -MDA5 antibodies that need further

characterization by IP-WB. The presence of several autoAgs of the same molecular weight needs further testing to differentiate the specificities [46, 47]. Additional autoAbs identified by protein-IP were anti-Ro/SSA in 5 cases, and in 3 cases a double positivity with anti-Jo-1 was present. By protein- and RNA-IP we were able to confirm the presence of two less common anti-synthetase antibodies compared to the more common anti-Jo-1: we identified one positive anti-EJ (anti-glycyl tRNA synthetase) and one anti-PL-12 (anti-alanyl tRNA synthetase) case, in a DM and a PM with anti-synthetase syndrome, respectively **(Figure 7, panel C).** By RNA-IP we identified the 7SL RNA band characteristic of anti-SRP antibodies, in association with anti-Ro/SSA, in a patient with PM. Eight cases still have no known anti-ENA specificity and in 4 also serum ANA is undetected by standard IIF. However, this may be due to technical issues such as fixation steps in the preparation of ANA slides, causing lack of reactivity during IIF, and not to the real absence of autoAbs also in this set of PM/DM patients.

**4- PSORIATIC ARTHRITIS (PsA).**

We tested by protein-IP and RNA-IP sera from 58 patients affected by PsA collected in the years 2013-2015 in our outpatient clinic. These patients were consecutively enrolled based on the negativity of serum RF and anti-CCP antibodies, and patients with different manifestation of PsA were included (i.e. peripheral arthritis and axial disease). Protein-IP allowed the identification of specific bands corresponding to anti-Ago2/Su in 2 cases, anti-Ki/SL in 2 cases and one weak anti-PM/Scl in PsA patients (**Figure 8, panels A and B**). None of these patients had clinical features suggestive for connective tissue disease in association with PsA. As shown in **Figure 8**, **panel B** we were able to see immunoprecipitated proteins of variable molecular weight (48-180kD), but these do not correspond to known autoAbs and no common band was detected in our subsets of PsA. One PsA patient later developed myositis, Raynaud's phenomenon and sclerodactyly but no specific autoantibody was identified by protein- and RNA-IP. Through RNA-IP we were able to identify one band corresponding to anti-SRP (7SL band) in one case, but no other specific autoantibody was

present in PsA patients *(data not shown).*These results indicate that PsA patients may have an autoimmune response similar to the one developed by RA seropositive patients, but no common autoAb has been identified so far to allow clinical correlations and validation as circulating biomarkers.

*LL37 in PsA patients.*

Besides testing our sera of PsA patients by protein- and RNA-IP to identify common autoAbs, we also focused our attention on the study of LL37 in our cohort, to verify the altered expression of this molecule also in patients affected by PsA and not only by PsO. We measured the concentration of LL37 in 31 PsA patients and 3 healthy controls, as shown in **Figure 9 panel A**. Results demonstrate a higher concentration of LL37 in PsA patients compared to controls, with the identification of 7 PsA patients (7/31, 22.5%) with high concentration of LL37 (>55ng/ml) compared to the other 24 PsA patients who do not express high concentration of LL37 (p=ns). We also compared the concentration of LL37 both in the serum and in the synovial fluid of three patients affected by PsA with active joint inflammation from which we performed knee arthrocentesis (**Figure 9, panel B**). Levels of LL37 were not different in serum and synovial fluid (p=ns), and similarly there was no difference in the control group represented by healthy people who underwent knee arthrocentesis for osteoarthritis, a disease considered degenerative and not inflammatory. These preliminary results may indicate that LL37 could be used as a diagnostic marker, but not as a marker of activity or severity of disease, as for anti-CCP antibodies in RA patients.

## 5- BEHÇET DISEASE (BD).

No known autoAb has been identified in sera of patients affected by BD, and for this reason, we analyzed by IP the sera of 45 BD patients collected by our collaborator Luca Cantarini (U.O.C. Reumatologia, Dipartimento di Scienze Mediche, Chirurgiche e Neuroscienze; Azienda Ospedaliera Universitaria Senese; Ospedale Santa Maria alle Scotte; Siena, Italy) to identify possible bands corresponding to new autoAgs recognized by BD patients. Only in a few cases we were able to see

bands of unknown antigens immunoprecipitated by our sera, but no common band was detected in the set of samples we tested, so we cannot support the presence of specific autoAbs and perform clinical correlations (**Figure 10, panels B and C**).

## DISCUSSION

Systemic autoimmune rheumatic diseases (SARDs) are characterized by the presence of serum autoAbs directed against cellular components belonging to different tissues and organs. The role played by innate and adaptive immunity in the etiology and pathogenesis of SARDs is still largely unknown, and most reports refer to polygenic and polyenvironmental trigger factors as the primum movens to the onset of SARDs. As for adaptive immunity that is established by cellular key players such as T cells and B cells, the mechanism of autoAbs production in response to recognition of self-antigens is still unknown and this leads to the breakdown of immune tolerance and autoimmunity. In fact, the target of autoAbs is represented by self-antigens that normally play physiological roles in cellular activities, but in some individuals the immune response does not recognize them as self and this triggers the autoimmune and inflammatory response. AutoAbs can be identified also in healthy individuals, and for this reason they are called natural autoAbs and they are well preserved in vertebrates to maintain immune homeostasis [125, 126]. These natural autoAbs are primarily of the IgM isotype and can bind to self antigens with low affinity and low specificity, and by doing so they seem to have a protective role from apoptotic and tumoral antigens [127]. On the other side, pathogenic autoAbs are continuously identified in patients affected by SARDs, and they are responsible for tissue and cell damage through several proposed mechanisms: (i) induction of cytotoxicity after autoAb binding, through complement or phagocytic mechanisms; (ii) autoAb binding and modulation of cell surface receptors; (iii) immune-complex mediated damage; (iv) cross-reaction between intracellular and membrane antigens; (v) penetration of autoAbs into living cells; (vi) binding to extracellular molecules [128-132]. The mechanisms through which autoAbs recognize one specific multicomplex antigen among thousands expressed by a cell is not exactly known. Several hypothesis have been postulated, and among them the one referring to the cryptic epitope has been recently reevaluated to explain autoAb production in PM/DM patients [37]. The change in the amino acid sequence of a protein may be responsible for the change of protein degradation and thus lead to the formation of cryptic epitopes that are non-self to the organism thus

triggering the autoimmune response [13, 14]. Despite the still unknown details in the onset of autoimmune response and SARDs as its clinical expression, the first step to recognize the presence of an autoimmune response in a specific clinical setting is represented by the identification of ANA through the standard method IIF which is used on a regular and screening setting worldwide [16]. As discussed in the introduction, serum ANA are directed against cellular nuclear components and since 1948 with the LE cell phenomenon in SLE patients, hundreds of ANA specificities have been described worldwide. In the last decade, several groups and several international workshops were dedicated to the development of a consensus on the nomenclature and pattern description, and this led to the publication of official reports in 2014 and 2015 [133, 134]. Several common and rare patterns have been identified using HEp-2 cells as substrate, with the need to increase the correct report of ANA results worldwide. In fact, it has been previously demonstrated that ANA may represent the first alarm for the onset of a SARDs, also in the absence of clinical features of diseases such as SLE [8]. However, serum ANA are not disease specific and they can be produced also for concomitant conditions such as infection or organ-specific autoimmune disease, one of the most common being autoimmune thyroid disease, and then they can become negative or low-titer when retested [17]. This was shown also in our recent publication on the analysis of ANA and anti-ENA in a selected population in Northern Italy, observed for a period of 15 years [18]. What makes the difference and increases the risk to be in front of a real case of SARDs is the high titer of ANA and some patterns that are more associated with clinical disease phenotypes such as ACA in SSc patients. These elements should bring physicians to ask for further testing as anti-dsDNA in the suspect of SLE if the ANA pattern is homogeneous, or to testing of anti-ENA in the suspect of other CTDs such as SSc and PM/DM when ANA are present in specific pattern such nucleolar. Several autoAbs have been identified and described since 1990s, and they are also included in the classification and diagnostic criteria as discussed above, but many of them are not available for the routine and screening commercially available tests. In diseases such as SLE and SjS no much progress has been done in the identification of new autoAbs as biomarkers, while in other

conditions such as SSc and PM/DM a lot of research has been done in the last years to identify new autoAbs. In order to define autoAbs as biomarkers able to trace the presence of an autoimmune disease and all the clinical correlations, it must be identified in a specific disease and it must be validated by studies based on its specificity, sensitivity, accuracy and reproducibility, so that all the clinicians worldwide may be able to identify specific autoAbs. One of the main problems in this validation of autoAbs as biomarkers is the small number of cases with a rare disease, and patients are spread worldwide, thus leading to bias due to enrolment, techniques of autoAb detection and validation of the results. As for autoAbs in PM/DM, these have been mainly discovered in the last decade [46-49, 135, 136] but none of them is included in the diagnostic or classification criteria except for anti-Jo-1 in PM patients affected by anti-synthetase syndrome [10]. Most recent autoAbs in SSc and PM/DM have been identified only in a research setting and are not included in routine autoimmunity tests with few exceptions at an international level, and this leads to additional problems such as (i) lack of standardization; (ii) reduced number of positive cases that could be studied for autoAbs clinical association; (iii) identification through time and labor consuming techniques such as IP; (iv) no commercially available technique such as ELISA. All these limitations lead to problems in the diagnosis, clinical evaluation, follow-up and therapeutic choices in CTD, because autoAbs can be used to characterize subsets of patients with different organ involvement. One of the most recent cases is represented by anti-RNAPIII antibodies in SSc with high risk of renal crises, that were tested in a research setting until 2006 when an ELISA kit became commercially available and it is now used for routine testing [64]. Similarly, we tested and provisionally patented a new method for detection of anti-U3/fibrillarin and -Th/To antibodies in SSc patients to allow faster and larger scale evaluation of these antibodies not only in a research setting [28]. This method uses a combination of standard RNA extraction by phenol/chloroform/isoamyl alcohol followed by reverse transcription and real-time PCR instead of running urea-PAGE gel and silver staining that can cause variable and just qualitative results. All these concerns address our **unmet need #1**, which is the lack of standard testing for most new

autoAbs in SSc and PM/DM patients, leading to mistakes in the patients' management that may be irreversible especially when affecting internal organs. For example, a recent autoAb called anti-MDA5 was identified in DM patients affected by rapidly progressive interstitial lung disease, so the lack of methods for anti-MDA5 identification could lead to worse prognosis and life-expectancy in anti-MDA5 positive DM patients [137-139]. On the other side, the presence of anti-Mi-2 antibodies in a DM patient is associated with good prognosis and quick response to immunosuppressive treatment for classical DM skin lesions, so the identification of anti-Mi-2 antibodies does not justify an aggressive treatment [140, 141].

These considerations also lead us to our **unmet need #2**, which is the need to increase the number of cases positive for rare autoAbs in different populations, in order to have a better description of the meaning of autoAbs in patients affected by SARDs and belonging to different ethnic groups. In fact, prevalence of autoAbs in conditions such as SSc and PM/DM can vary largely according to the ethnic group in which it was identified, as we also demonstrated in previous studies performed in the Italian population [33, 46, 47, 142, 143]. For these reasons we decided to set up in our laboratory the two methods, protein and RNA-IP, that are considered gold-standards for the identification of known and unknown autoAbs, and to study the group of patients followed at our outpatient clinic. The main reasons to perform IP are: (i) the capacity of this technique to screen almost all known autoAbs performed by a single assay; (ii) the visual and qualitative confirmation of the reactivity of the tested human serum to the target antigen, contrary to methods such as ELISA in which the result may not reflect the reactivity with the actual target; (iii) antigens in IP are more close to the native condition compared with antigens used in other immunoassays (i.e. western blot, ELISA) and this is important for the identification of autoAbs that recognize multiproteins or multiprotein-nucleic acid complexes; (iv) protein- and RNA-IP are fundamental for the identification of rare autoAbs without commercial and routine assays to be tested, in association with the mass spectrometry analysis of the IP component. To increase the number of cases affected by PM/DM identified in Europe for their better evaluation from several points of view, a registry

called “Euromyositis” was created in 2010 and we were admitted to this group in 2015 to add data of our patients for a better clinical and laboratory evaluation of myositis cases in Europe (www.euromyositis.eu).

Despite the advantages just mentioned, protein- and RNA-IP also have several disadvantages and for these reasons they are performed only in a few laboratories worldwide, mainly in a research setting, and they are not suitable for routine testing. In fact, they are time consuming and it takes weeks to have results; they are labor consuming because most buffers and material are home-made and not commercial; and it requires dedicated space and instruments for the use of high concentration of $^{35}$S-radioactive labeling for protein-IP (14mCi). The antigen cellular source commonly used is represented by K562 cells because they grow rapidly and in suspension [144], but alternative cell lines that can be used (i.e HeLa and HEp-2 cells) expressing the same antigens require more work and longer time to be radiolabeled in a large quantity.

In our research laboratory we were able to set up protein- and RNA-IP and we first tested known reference sera as internal positive controls. These samples were CDC ANA reference sera provided through the website www.autoab.org that sends one vial of 19 samples to be used for as standard by laboratories worldwide. We are now part of the group called ANA standardization committee that aims at creating standards for ANA testing and identification following the same rules in all the autoimmunity laboratories worldwide, and in this group new techniques are still compared to IP as standard. After testing reference sera for known autoAg, we had the confirmation that IP was correctly performed, and in particular that the preparation of all buffers was done properly, the radiolabeling of K562 cells and their growth status were performed correctly as demonstrated by the identification of antigens such as phosphorylated topo I that is produced by well-growing K562 cells.

After verifying the correct performance of protein- and RNA-IP we decided to test our human samples of patients affected by SARDs. First, we tested human sera which had no-known anti-ENA despite ANA positivity, because this condition is highly suspicious for presence of an autoAb that

cannot be recognized by commercial techniques. As expected, SSc and PM/DM patients expressed several autoAbs that are considered rare and that need further characterization, as for the anti-140k band antibodies that may be MJ or MDA5 and for this we need further testing by IP-WB. In a subset of SSc patients reported as ACA despite having a complex and severe SSc we were able to see a new IP pattern that was recognized as the one shown in WB by hnRNPs proteins [119]. We tested two anti-hnRNP autoAbs based on their molecular weight, hnRNP C1+C2 and hnRNP L, but only hnRNP L showed reactivity when performing IP-WB as shown in our results section. This is a new pattern that needs to be further tested using other anti-hnRNP antibodies, and that may be associated with specific centromere proteins as we are planning to demonstrate by performing ELISA of specific CENP- proteins. If this is true, we cannot simply say that ACA positivity is associated with limited SSc, but in this cathegory we can also recognize other specific features of more aggressive vasculopathy in limited SSc, and we can conclude that not all SSc autoAbs are mutually exclusive.

Another interesting set of SSc cases showed a pattern that we already observed in patients affected by PBC (*unpublished data*), represented by a set of four bands of molecular weight 75-50-40-34kD. When we analyzed retrospectively these six positive samples, we confirmed our association with the ACA pattern in all of them, and we also identified 2/6 cases with reported AMA positivity by routine clinical testing and a diagnosis of PBC already performed and treated. These results open to the possibility that an AMA pattern can be identified also by protein-IP, and this could help in the early diagnosis of PBC in SSc patients, a condition often underestimated until the appearance of clinical or laboratory features of PBC [145].

As mentioned in **unmet need #2**, the identification of autoAbs by uncommon techniques such as IP needs to be increased in the research or routine centers performing autoimmunity tests, in order to increase the number of positive cases identified for specific autoAbs and to know the exact prevalence of autoAbs and their meaning. Recently, one autoAb identified in statin-induced

myositis, called anti-HMGcoA reductase antibody, was first described as a 200/100kD doublet by IP and recent efforts have been done to create an ELISA for its routine testing [135, 146-149].

Among the limitations of IP, we can include the fact these techniques cannot identify several proteins based on their molecular weight if too low or too high, and among them citrullinated or carbamylated proteins present in RA patients cannot be identified by IP. This may be the first reason why we were not able to find results in our human sera of patients affected by PsA and also in those affected by a specific vasculitis such as BD. One of the main objectives of using IP in PsA and BD was to demonstrate the presence of reactivity and thus autoimmune activity in these conditions that have often been described as chronic or autoinflammatory rather than autoimmune. Our results show that there is no specific pattern identified by IP in these two conditions, and thus we were not able to identify new biomarkers probably because of technical limitations already mentioned or because the mechanisms underlying the disease are not autoimmune but really inflammatory and they do not lead to autoAb formation. In the case of PsA, in the last ten years much interest has been brought to the role played by the molecule LL37 as an antigen recognized by T cells in PsO [89, 150]. This antigen is highly expressed in PsO plaques and skin alterations and it is correlated with severity of PsO, while no data have been reported on LL37 and PsA. Starting from the hypothesis that B cells and autoAbs may not be the key players of the inflammatory response in PsA patients, as shown by our negative IP results, we hypothesize that also in PsA patients and not only in PsO LL37 may be the main antigen triggering a T-cell mediated response. In fact, as described in the Results, in our cohort of patients we were able to identify 7 PsA cases expressing high concentration of LL37 even if not statistically significant compared to controls, somehow mirroring the partial penetrance of LL37 T cell reactivity reported by Lande and Colleagues [150]. These results support a possible role of this antigen also in PsA patients, and we aim to further study the role of LL37 by increasing the number of patients and controls, and to evaluate possible correlations with specific disease features (i.e. peripheral arthritis versus spondiloarthritis, organ involvement versus pure joint inflammation). Another aspect shown by our

experiments is that LL37 concentration does not change in the serum compared to synovial fluid obtained from the same PsA patients during active inflammation states, and this means it should not be considered as an activation marker but rather a disease marker. One similarity could be with anti-CCP antibodies in RA, which are now considered markers of early diagnosis of erosive forms of arthritis but they are not used to follow the disease overtime. Another possible application of IP could be the use of an antigenic source different from K562 cells, such as a cell line derived from melanoma or skin cells that could express the antigenic target of PsA. The disadvantage of IP with this variation is that the cellular line needs to grow quickly and with high concentration to be as effective as K562 cells normally used for IP.

Our results support the possibilities of setting up a technique such as IP in a research setting to diagnose SARDs without bias due to recent new automatic and commercial techniques, which is important in the view of “translational medicine” in order to “translate” research results to real-life clinical settings.

## FINAL KEY MESSAGES

- Protein and RNA-IP can be set up using specific reference samples
- These techniques can be used for the identification of known and unknown autoAbs in SARDs
- Protein--IP allowed the identification of a new pattern of different molecular weight bands (140/40-25kD) in a subset of SSc patients affected by limited SSc with severe vascular disases
- Our IP-WB confirmed that sera with positive anti-140/40-25kD pattern react to hnRNP-L antibodies
- Protein-IP allowed the identification of a pattern (75-50-40-34kD proteins) corresponding to AMA in SSc patients affected by PBC
- Protein- and RNA-IP can be used together with routine laboratory tests for the early diagnosis and better follow-up and treatment of SARDs patients
- LL37 could be a promising diagnostic antigen not only in PsO but also in PsA patients, as shown by positive samples identified in our cohort by ELISA, but it is not a marker of PsA activity
- IP can be used in the setting of “translational medicine” to improve diagnosis and thus management of SARDs patients

**FIGURES.**

**Figure 1. Classification and diagnostic criteria of SARDs including autoAbs. Panel A**. Main subsets of systemic autoimmune rheumatic diseases (SARDs), divided in three groups: connective tissue diseases, arthritis and vasculitis. **Panel B.** List of autoAbs currently included in the diagnostic criteria of SARDs, as reported by the references shown in the figure.

Abbreviations: RA, rheumatoid arthritis, PsA psoriatic arthritis, SLE Systemic Lupus Erythematosus, SJS Sjögren's syndrome, SSc Systemic Sclerosis, PM Polymyositis, DM Dermatomyositis, UCTD Undifferentiated connective tissue disease, MCTD Mixed connective tissue disease.

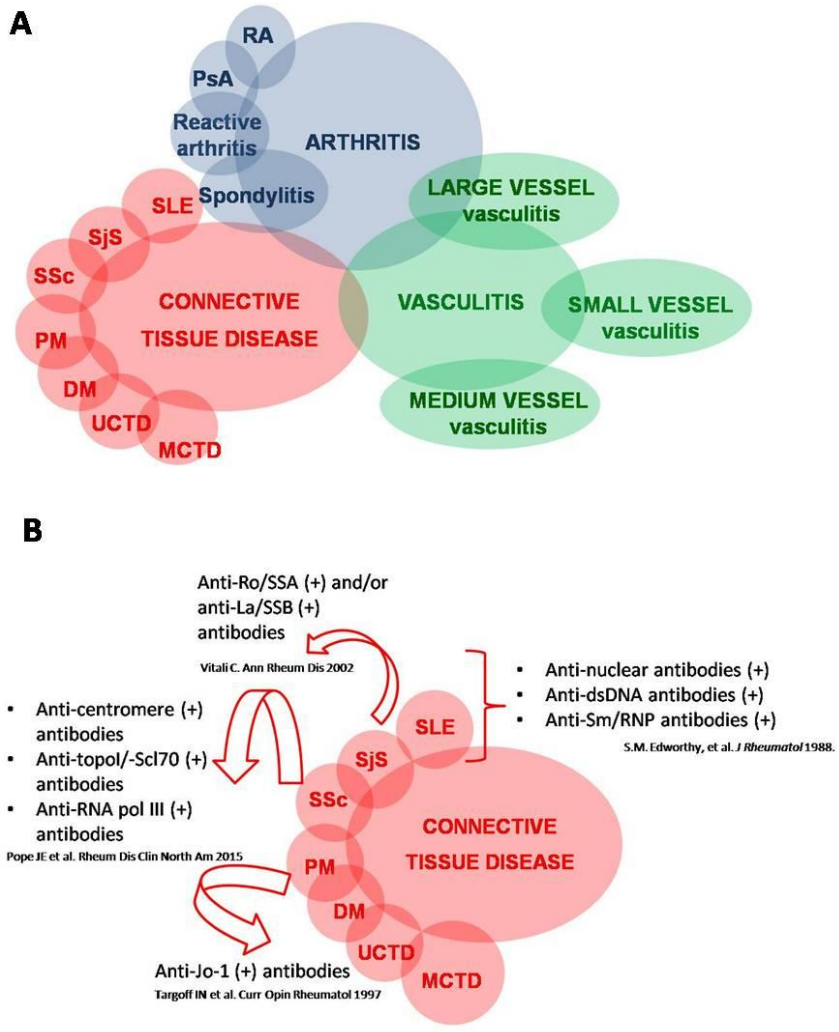


**Figure 2. Patients' sera analyzed by protein- and RNA-IP in the present study** (see section Patients and Methods for details).

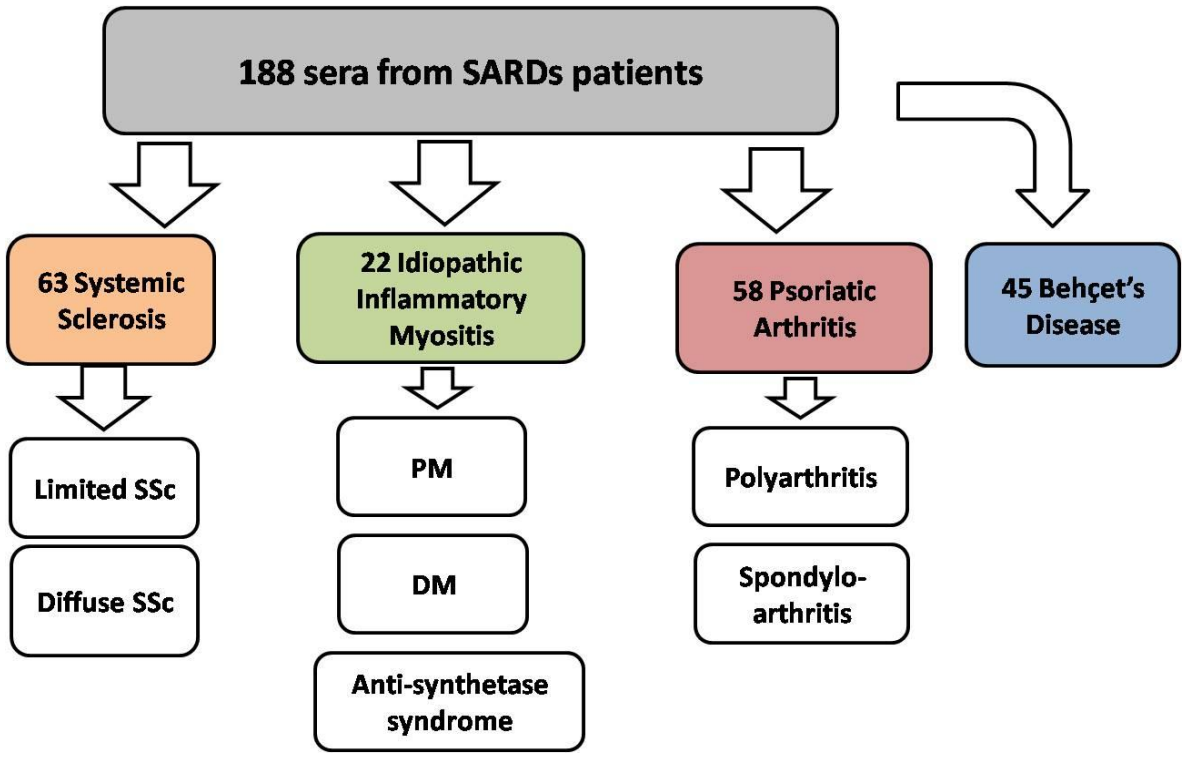


**Figure 3. Comparison of standard sonication with alternative methods to obtain cell lysate from $^{35}$S- radiolabeled K562.** The reference sample was the anti-U1RNP+ Ro/SSA+La/SSB positive serum obtained from CDC ANA reference sera (www.autoab.org). We compared standard sonication (45 seconds twice, with one minute interval), with other two methods: (i) vortexing for 60 seconds for 3 times, with the pellet in ice between the cycles to avoid heating; (ii) 5 cycles of freezing in dry ice and thawing in 37°C waterbath for 1 minute at each step. As shown in the figure, results are comparable and we decided to use vortexing instead of sonication for protein-IP radiolabeled K562 cell extract.

**Figure 4. Reference sera. Panel A.** Identification of reference sera by protein-IP (8% SDS-PAGE gel, fluorography and autoradiography); anti-Ro/SSA can be identified by the presence of the Ro60kD antigen with smiling shape, anti-La/SSB by the 48kD antigen and anti-topo I by the 110kD antigen with phosphorylated smear; NHS (normal human serum) is the negative control**. Panel B.** Identification of reference sera by RNA-IP (12% urea-PAGE gel, silver staining). Anti-Sm reference serum is recognized by the immunoprecipitation of U2-U1-U4-U5-U6 RNAs; U1+Ro/SSA reference serum immunoprecipitates the U1 band plus the Y-RNAs that characterize the Ro complex antigen; total RNA is the standard used as positive control, and negative controls (neg) are also run and they do not show RNA bands.

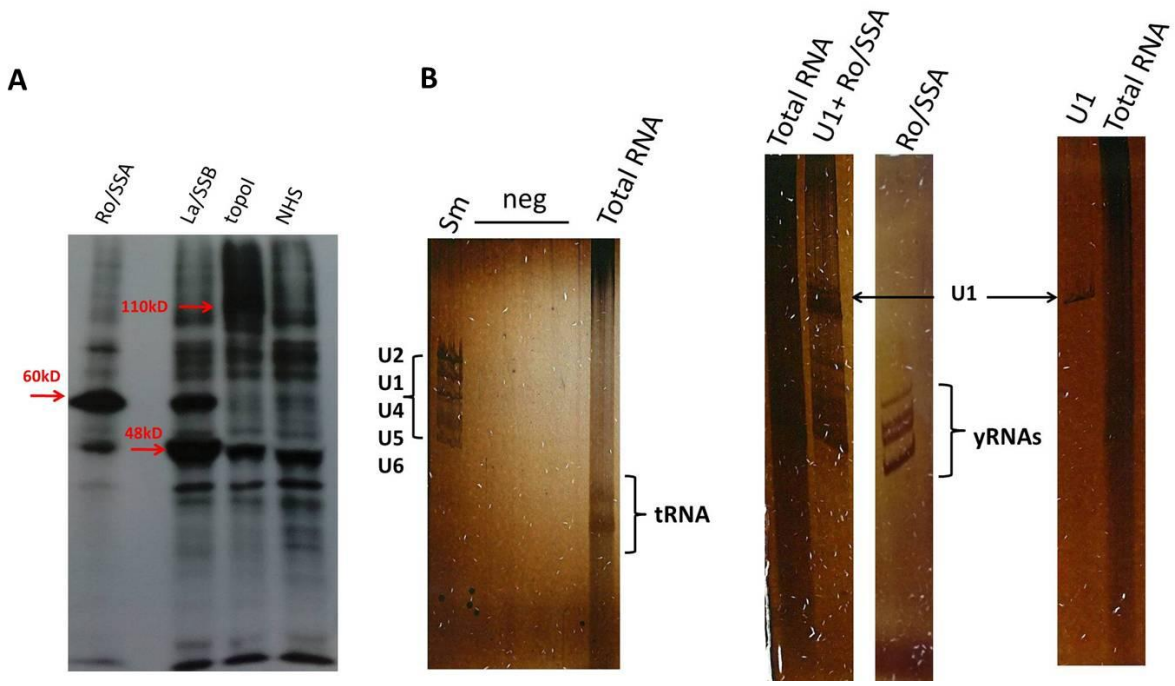


**Figure 5. Systemic sclerosis. Panel A.** The bar graph shows the autoAb and the number of corresponding cases identified in SSc patients ACA (-) and topoI (-). **Panel B.** Anti-RPA positive SSc patient, recognized by the heterotrimer 70/32/14 kD shown by the arrows (Protein-IP,12% SDS-PAGE gel). **Panel C.** RNA-IP (12% urea-PAGE gel, silver staining) showing representative positive SSc cases for the specificities described in the figure. **Panel D.** Protein-IP showing a new pattern (indicated by squares and arrows), identified in 8 SSc patients. **Panel E.** IP-WB of 6 SSc cases positive for hnRNP L identified in the group of anti-140/40-25 kD SSc cases.

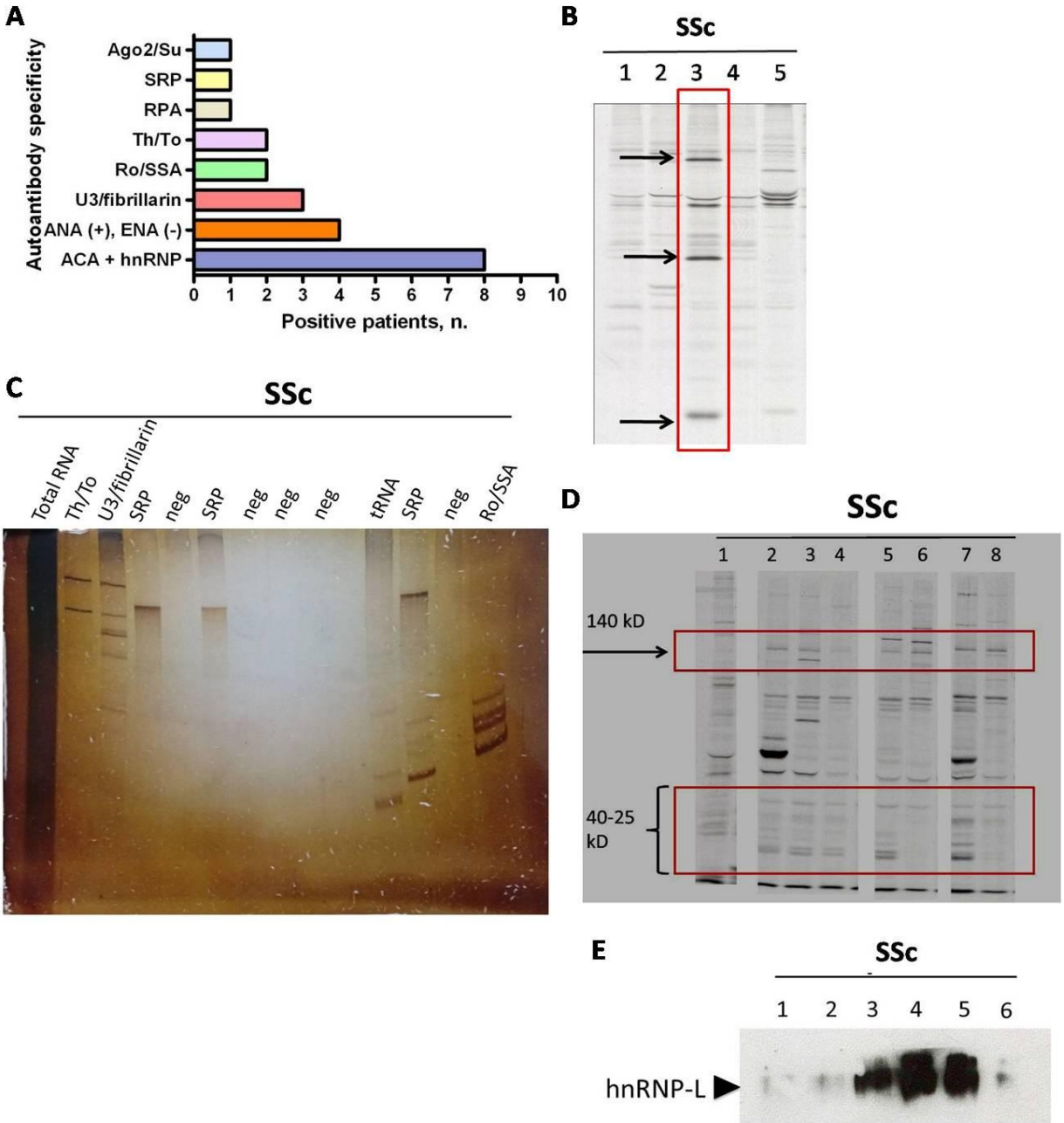


**Figure 6. Systemic sclerosis. Panel A.** Protein-IP results of a set of SSc patients, with positive autoAgs described in the figure. **Panel B.** Protein-IP results of a set of SSc patients, including two samples with bands of 75-50-40-34k that are referred as "AMA" positive and shown in the red square. **Panel C.** Protein-IP results of a set of SSc patients, including one AMA (+) in the red square. **Panel D.** Protein-IP results of a set of SSc patients, with three additional AMA (+) samples in the red square.

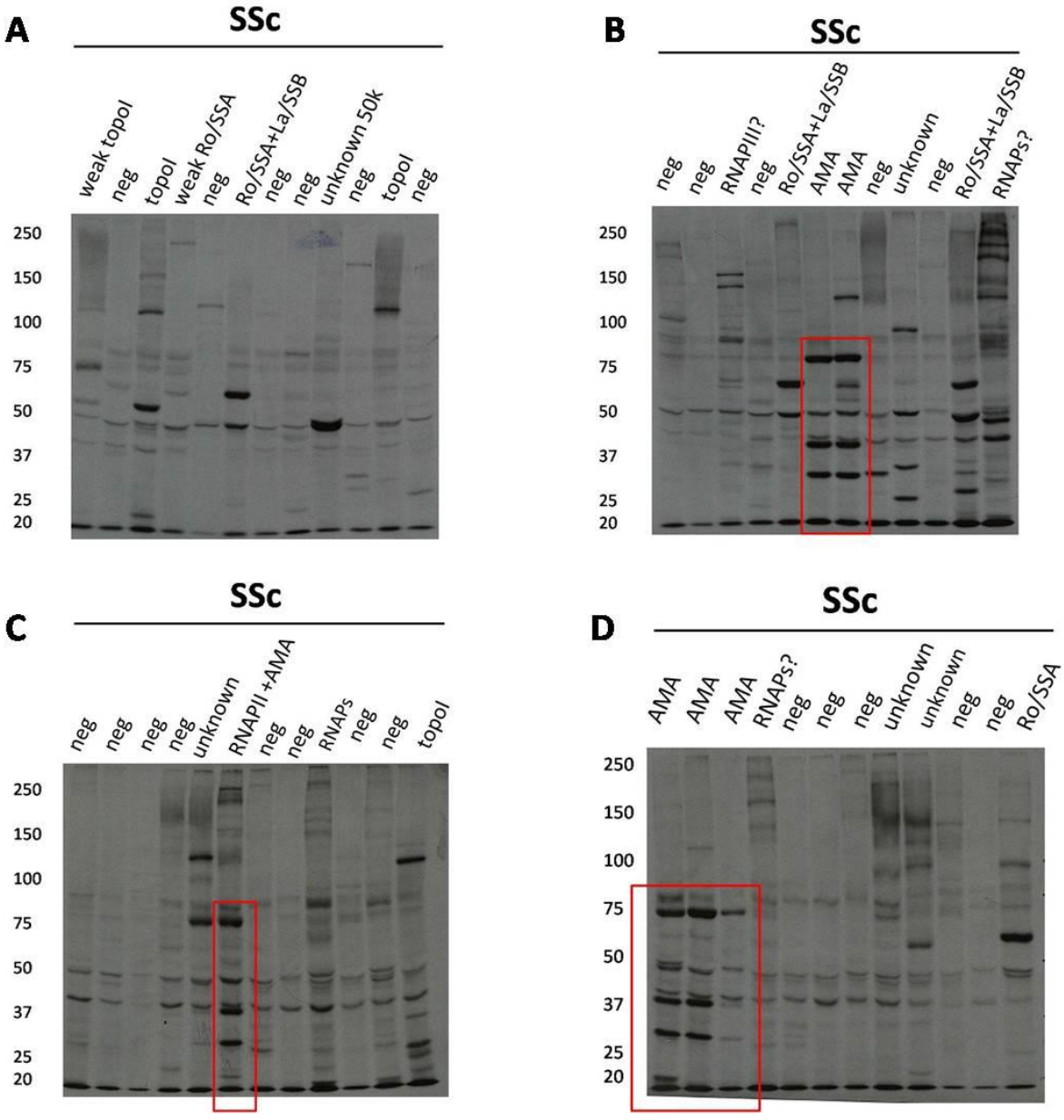


**Figure 7. Polymyositis/dermatomyositis. Panel A.** Bar graph showing the autoAb and the number of corresponding cases identified in PM/DM patients. **Panel B.** Protein-IP (12% SDS-PAGE gel) of representative PM/DM patients and corresponding autoAb, as shown by the arrows and by the associated antigen (in red) **Panel C.** RNA-IP (12% urea-PAGE gel, silver staining) of representative positive PM/DM cases, including one anti-PL-12 (+) case in a patient affected by anti-synthetase syndrome. Reference sera are indicated by the asterisk*.

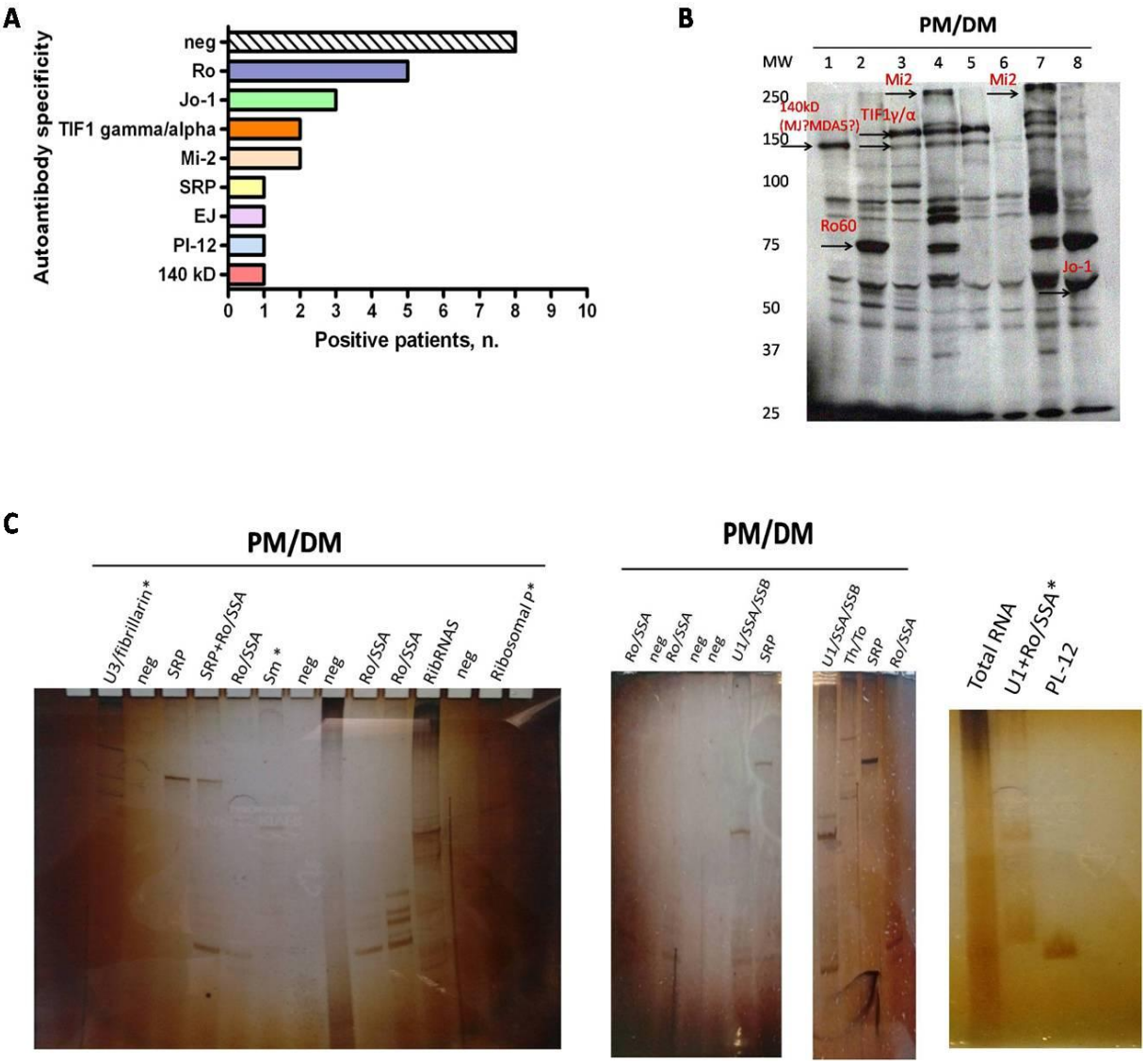


**Figure 8. Psoriatic arthritis. Panel A**. Protein-IP of PsA patients' sera, where we can recognize autoAbs such as anti-Ago2/Su and Ki/Sl based on the molecular weight as shown. **Panel B.** Additional positive PsA cases with unknown specificity, indicated by the arrows.

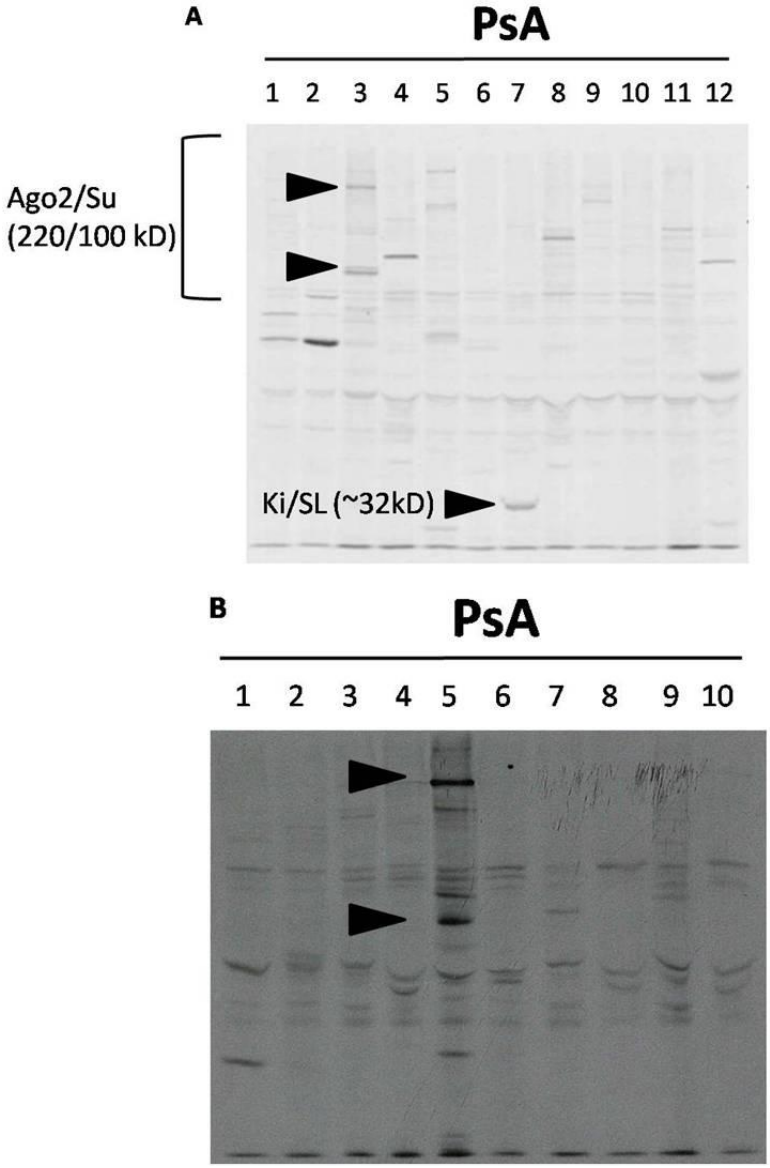


**Figure 9. LL37 in PsA. Panel A.** Positive LL37 cases compared to controls (p=ns); the cut-off for positive LL37 samples was established at >55ng/ml (mean ± 3 standard deviations). **Panel B.** Comparison of LL37 levels in synovial fluid (SF) versus serum of three PsA patients with active disease (p=ns); the same comparison was also performed in SF from osteoarthritis (OA) patients and in serum of healthy controls (HC, p=ns).

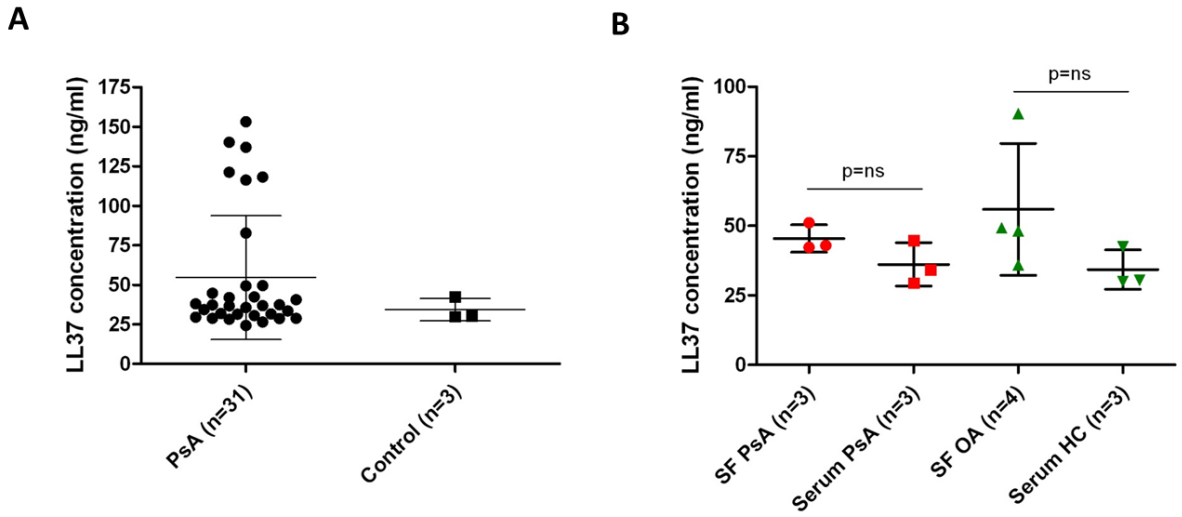
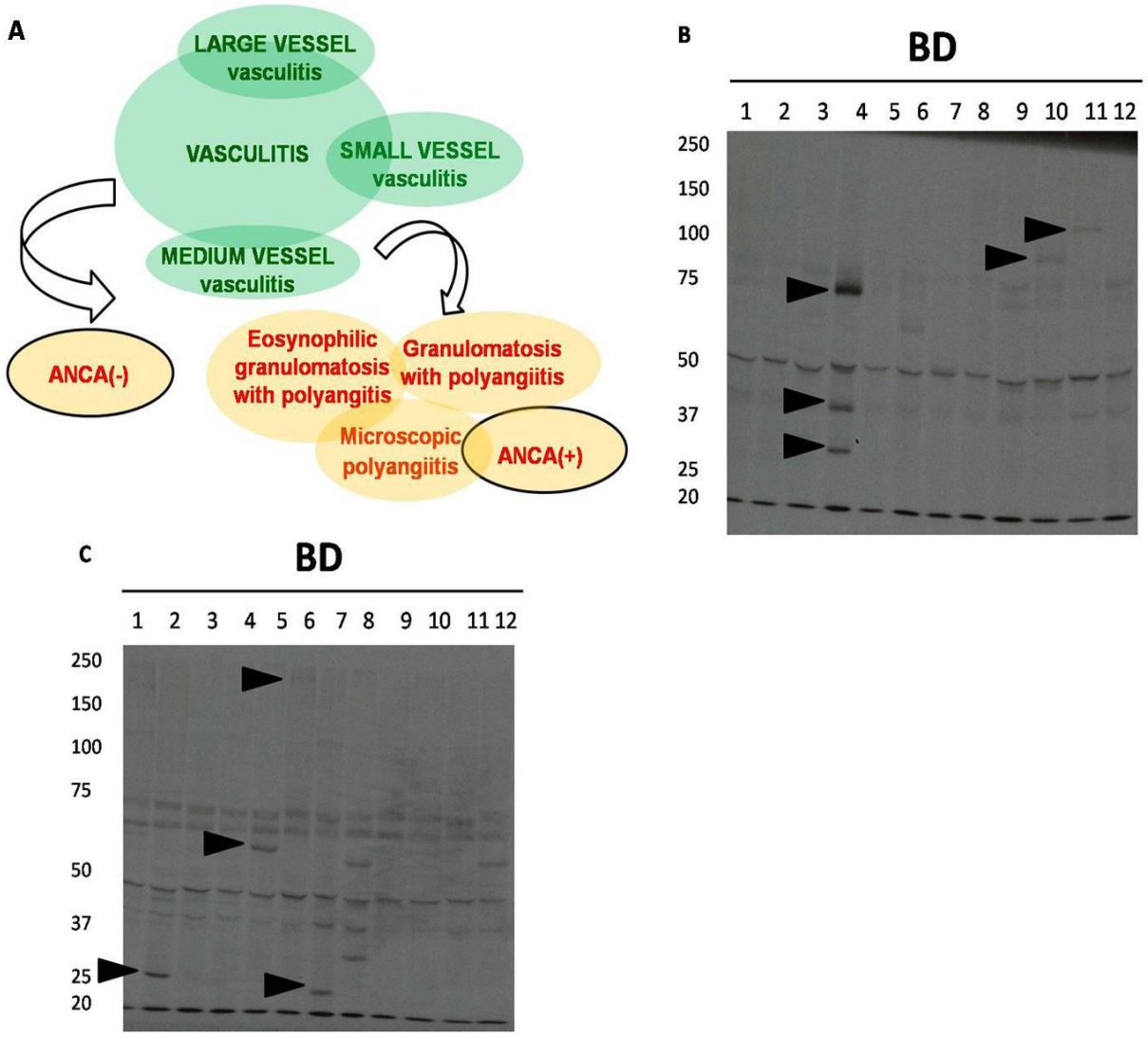


**Figure 10. Behcet disease (BD). Panel A.** Description of the present classification of vasculitis, that are divided based on the size of the affected vessels (small, medium, large) and to the positivity or not of ANCA. BD belongs to the group of ANCA (-) vasculitis, only three vasculitis can be diagnosed based on the presence of ANCA with different patterns (MPO/PR3). **Panel B**. Representative results of protein-IP for BD patients (8% SDS-PAGE gel). Arrows indicate bands recognized as autoAg by protein-IP, but no common band was identified in our set of samples. **Panel C**. Another example of representative samples tested by protein-IP (8% SDS-PAGE gel). Also in this panel arrows indicate bands detected by IP but no common band was identified.



## TABLES

**Table 1. AutoAbs in SSc and PM/DM that can be identified by protein- and RNA-IP according to the characteristics of the antigen described in the table.**

| SSc AutoAb | Target autoAg | Protein-IP | RNA-IP |
|---|---|---|---|
| **ACA** | CENP proteins | - | - |
| **Topo1 (Scl70)** | Topoisomerase I | 110kD with phosphorylation smear | - |
| **RNAPs** | RNA polymerase I/II/III | Bands at 240-220 and 145kD (RNAPII), 197 and 126kD (RNAPI), 155 and 138kD (RNAPIII) | - |
| **Th/To** | MRP complex | - | 7-2 and 8-2 RNA |
| **U3/fibrillarin** | fibrillarin | 34kD | U3RNA |
| **PM/Scl** | PM75 and PM 100 protein | 100kD | - |
| **PM AutoAb** | | | |
| **Jo-1** | Histidyl tRNA synthetase | 50kD | $tRNA^{his}$ |
| **PL-7** | Threonyl tRNA synthetase | 80kD | $tRNA^{thr}$ |
| **PL-12** | Alanyl tRNA synthetase | 110kD | $tRNA^{ala}$ |
| **EJ** | Glycyl tRNA synthetase | 75kD | $tRNA^{gly}$ |

| | | | |
|---|---|---|---|
| **SRP** | Signal Recognition Particle | 72, 68, 54, 19, 14, 9kD | 7SL RNA |
| **DM AutoAb** | | | |
| **Mi-2** | Helicase protein | 240, 150, 72, 65, 63, 50 and 34kD | - |
| **MDA5 (CADM140)** | MDA5 (melanoma differentiation-associated gene 5) | 140kD | - |
| **MJ/NXP-2** | NXP2 (MORC3) | 140kD | - |
| **TIF1γ/α** | TIF1γ/α | 155 and 140kD | - |
| **TIF1β** | TIF1β | 120kD | - |
| **SAE1,2** | Small ubiquitin-like modifier 1 (SUMO-1) activating enzyme | 90 and 40kD | - |
| **Other AutoAbs** | | | |
| **Ro/SSA** | Ro60 | 60kD | Y-RNAs |
| **La/SSB** | La | 48kD | Y-RNAs |
| **Ago2/Su** | Ago2 | 200 and 100kD | - |
| **Ki (SL)** | Ki | 32kD | - |
| **U1RNP** | U1 ribonucleoprotein | - | U1RNA |

**Table 2. Main differences in the protocol for protein-IP versus RNA-IP.**

| Main procedure steps | Protein-IP | RNA-IP |
|---|---|---|
| K562 radiolabeling by $^{35}S$ | yes | no |
| SDS-PAGE gel | yes | no |
| urea-PAGE gel | no | yes |
| extraction by phenol/chloroform/isoamyl alcohol | no | yes |
| fluorography | yes | no |
| autoradiography | yes | no |
| silver staining | no | yes |

**Table 3. Rare autoAbs identified by protein and RNA-IP in SSc patients, excluding ACA and anti-topoI antibodies. Some autoAbs were identified by routine immunoblotting (IB) but not confirmed by IP.**

| SSc rare autoAbs | Routine IB (n.) | Protein-IP (n.) | RNA-IP (n.) | IIF pattern |
|---|---|---|---|---|
| Anti-RNAPIII | 1 (Rp155) | - | - | cytoplasmic |
| Anti-U3/fibrillarin | - | - | 3 | speckled+nucleolar |
| Anti-Th/To | 1 | - | 2 | speckled+nucleolar |
| Anti-SRP | - | - | 1 | cytoplasmic |
| Anti-Ago2/Su | - | 1 (+ topoI) | - | homogeneous+nucleolar |
| Anti-Ro/SSA | 3 | 3 | 2 | homogeneous and nucleolar |
| Anti-RPA | - | 1 | - | ACA |
| Anti-NOR90 | 1 | - | - | speckled+nucleolar |
| Anti-PM/Scl | 1 (PM100) | - | - | nuclear dots |
| Anti-Ku | 1 | - | - | speckled |
| 140-40/25kD pattern | - | 8 | - | ACA |
| AMA | - | 6 | - | ACA |

**Table 4. Rare autoAbs identified by protein and RNA-IP in PM/DM patients.**

| AutoAb | Diagnosis | Routine IB (n.) | Protein-IP (n.) | RNA-IP (n.) | IIF pattern |
|---|---|---|---|---|---|
| Anti-Jo-1 | PM+ ASS | 2 | 3 | 3 | speckled and cytoplasmic |
| Anti-PL-12 | ASS | 1 | 1 | 1 | speckled+cytoplasmic |
| Anti-SRP | PM | - | - | 1 (with Ro/SSA) | cytoplasmic |
| Anti-Mi-2 | DM | - | 2 | - | homogeneous and speckled |
| Anti-EJ | DM | - | 1 | - | n/a |
| Anti-TIF1γ/α | DM | - | 2 | - | speckled |
| Anti-Ro60 | PM/DM | 5 | 5 | 5 (3 with anti-Jo-1) | speckled |

**List of abbreviations.**

ACA, anti-centromere antibodies
ACR, American College of Rheumatology
AMA, anti-mitochondria antibodies
ANA, anti-nuclear antibodies
ANCA, anti-neutrophil cytoplasmic antibodies
APCs, antigen-presenting cells
ASS, anti-synthetase syndrome
autoAb, autoantibody
autoAg, autoantigen
BD, Behçet disease
CCP, cyclic citrullinated peptide
CRP, C reactive protein
CTDs, connective tissue diseases
DM, dermatomyositis
ELISA, enzyme-linked immunosorbent assay
ENA, extractable nuclear antigens
ESR, erythrocyte sedimentation rate
hnRNP, heterogeneous nuclear ribonucleoproteins
IB, immunoblotting
IIF, indirect immunofluorescence
IP, immunoprecipitation
IP-WB, immunoprecipitation-western blot
MDA5, melanoma differentiation associated gene 5
NHS, normal human serum
NXP-2, nuclear matrix protein 2
PAS, protein A Sepharose
PBC, primary biliary cirrhosis
PM, polymyositis
PsA, psoriatic arthritis
PsO, psoriasis
RA, rheumatoid arthritis
RF, rheumatoid factor
RNApol, RNA polymerase
RNA-IP, RNA immunoprecipitation
RPA, replication protein A
RPILD, rapidly progressive interstitial lung disease
SAE, small ubiquitin-like modifier activating enzyme
SARDs, systemic autoimmune rheumatic diseases
SLE, systemic lupus erythematosus
SjS, Sjögren's syndrome
SRP, signal recognition particle
SSc, systemic sclerosis
TIF1, transcription intermediary factor 1
UCTD, undifferentiated connective tissue disease

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