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Review Article

Pitfalls of autoimmune serological markers and practical diagnostic workflows in connective tissue diseases and rheumatoid arthritis

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ABSTRACT

Laboratory markers represent a highly valuable tool to support the diagnosis of systemic autoimmune diseases. However, their correct interpretation is essential to avoid misdiagnoses, with particular attention to the most common errors in their clinical use. It is also crucial for clinicians to understand when serological autoimmune markers should be requested, in order to reduce unnecessary, inappropriate, or costly testing for the healthcare system. A diagnostic approach based on the formulation of the right clinical questions is necessary to ensure the appropriate use of immunological markers. This review focuses on the most commonly used and requested autoimmune markers, with particular emphasis on connective tissue diseases and rheumatoid arthritis. Furthermore, common pitfalls and a diagnostic workflow are proposed to assist clinicians in the evaluation of patients with suspected systemic autoimmune diseases.

1. Introduction

The diagnosis of any disease typically involves, among others, essential components such as (1) medical history, physical examination, and observable clinical signs; (2) diagnostic tools, which today encompass various methodologies including laboratory tests, imaging, and others; and (3) clinical symptoms. These components can be expanded to achieve a more comprehensive diagnostic assessment, particularly in the context of complex or rare diseases.

Laboratory markers thus represent only one of the multiple elements necessary for establishing a diagnosis and must be interpreted within the context of their limitations.

Nevertheless, even laboratory markers alone, when carefully analyzed and interpreted with appropriate expertise, can offer highly informative and sometimes early diagnostic clues. This is especially true in the field of autoimmunity. Markers such as autoantibodies provide crucial support in fulfilling the classification criteria for systemic autoimmune diseases, thereby guiding clinicians toward an appropriate diagnostic evaluation [1]. Correct interpretation, however, is essential [2]. In specialties outside of rheumatology or clinical immunology, it is important that clinicians understand which markers to request, and when to refer a patient to the specialistto avoid the dual pitfalls of prematurely excluding autoimmune disease on the one hand, or making excessive and unnecessary testing requests on the other, potentially generating costs that are unjustified for the healthcare system [3].

This narrative review therefore aims to serve as a practical guide for clinicians, particularly internists, who are evaluating whether a patient may have a systemic autoimmune disease. Literature was identified through non-systematic searches of PubMed and EMBASE. Given the complexity and breadth of the topic, this article will focus primarily on connective tissue diseases (CTDs) and rheumatoid arthritis (RA), illustrating the role of autoantibodies and a selection of laboratory markers commonly used in everyday clinical practice. Also, this review will not cover the complexity and modernity of the new autoantibodies landscape that is emerging in research with extremely interesting associations between serological markers and autoimmune disease clinical features or prognosis, as for example in systemic lupus erythematosus (SLE) or other CTDs [4].

2. Connective tissue diseases

Autoantibodies are predominantly IgG-class antibodies that are mistakenly directed against self-antigens and are produced by differentiated B cells, the plasmacells [5]. A wide range of autoantibodies

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have been identified to date, making the interpretation of their positivity or negativity highly complex [1]. A preliminary consideration is whether it is appropriate to perform autoantibody screening in the general population (i.e., individuals considered healthy). At present, given current technologies and knowledge, mass screening is not recommended [6].

3. ANA: titer, pattern and brief overview of laboratory methods

Focusing specifically on antinuclear antibodies (ANA), they were first reported in the discovery of Lupus Erythematosus cells in 1948 [7]. Approximately 10–20% of healthy individuals may test positive for ANA depending on the titer [8–10]. It remains unclear whether these individuals will subsequently develop an autoimmune disease. Indeed, some autoantibodies can be detected several years before the diagnosis [11]. Moreover, ANA positivity may arise in the context of pharmacological treatments, infectious episodes, or neoplastic conditions [12]. A recent study, investigated the genetic contribution to asymptomatic ANA positivity through a genome wide association study on 1955 subjects finding that asymptomatic ANA positivity appears to have a heritability of roughly 25%. Interestingly, these ANA-positive but symptom-free individuals do not carry a greater genetic risk for lupus than those who test ANA negative [13]. Therefore, ANA testing and autoantibody testing in general should be considered as a secondary screening tool when there is already clinical suspicion of a systemic autoimmune disease (e.g., CTDs, idiopathic inflammatory myopathies, etc.). Also, ANA sensitivity and specificity varies over their positivity in the different CTDs [14].

An interesting aspect, is also about ANA nomenclature. Indeed, these autoantibodies, although named "antinuclear," can target various nuclear structures as well as extranuclear components. In fact, the International Consensus on ANA Patterns (ICAP) has proposed renaming them "anti-cell antibodies"; however, this terminology has not been widely adopted in clinical practice [15,16].

The main method used for ANA detection is the indirect immunofluorescence (IIF) assay, particularly on HEp-2 cells, a human laryngeal carcinoma cell line [17]. These cells are well-suited for the assay due to their high nucleus-to-cytoplasm ratio and the expression of numerous cellular antigens. For further details on the IIF method, we refer to specific literature [18]. Briefly, patient serum is incubated on a slide containing HEp-2 cells; if the serum contains antibodies targeting HEp-2 antigens, these will bind to nuclear (or other cellular) structures. Following a washing step, a fluorescently-labeled secondary antibody capable of recognizing human immunoglobulins is added. The slide is then examined under a fluorescence microscope. This final step highlights the crucial role of operator expertise in the interpretation of results obtained by this technique

Diagnostic interpretation of ANA should be guided by careful evaluation of both the titer and the fluorescence pattern observed. These two parameters are essential and can guide further testing or suggest a preliminary diagnosis [19]. The titer is expressed as a ratio, obtained by serial dilution of the patient's serum in saline. For example, a result of 1:320 indicates that ANA are still detectable when one part of serum is diluted with 320 parts of saline. The initial screening dilution is typically 1:80 and, based on common clinical practice, a titer of ≥1:160 is considered positive while a titer of ≥1:640 is classified as high [20] (See Table 1). Indeed, a titer of ≥1:160 and higher is a threshold considered valid to distinguish true positive among healthy subjects [9,21]. It is also important to note that based on EULAR/ACR 2019 SLE classification criteria, in SLE, ANA positivity is an entry criterion with a titer ≥ 1:80 [22]. A systematic review and metaregression on data of about 13000 SLE patients, identified that a titer of ANA 1:80 showed a sensitivity of about 98% in detecting SLE, enough to be considered an entry criterion for SLE [23]. Based on this premise, subjects with a low ANA titer should be considered by careful evaluation of the clinician that will base further investigations based on clinical symptoms.

Table 1
Common pitfalls to be aware when asking autoimmune serological markers.

	POSSIBLE PITFALLS
ANA	ANA by IIF (Indirect Immunofluorescence) target any part of the nucleus but can also be positive due to autoantibodies targeting extra-nuclear structures [16] Titers below 1:160 are are usually considered negativee [20] Based on EULAR/ACR 2019 SLE classification criteria, in SLE, ANA positivity is an entry criterion with a titer ≥ 1:80 [20] 10-20% of healthy individuals are ANA positive, usually representing a normal finding or a future autoimmune disease [9,10] ANAs can be positive for drugs, infections and cancer [12] Usually, there is no need to repeat ANA over the course of an already diagnosed CTDs, since ANA tend to be stable positive and the titer does not correlate with disease severity or progression Thyroid autoantibodies, specifically anti-thyroid peroxidase and anti-thyroglobulin antibodies may occasionally serve as the sole explanation for ANA positivity [55]. Do not miss to check them
ENA	Perform when ANA positivity in IIF is ≥1:160, or when ANA is negative but there is a strong suspicion of systemic autoimmune disease [37] Besides the standard panel, specific panels exist (the liver-specific ENA profile, the scleroderma ENA profile, or the myositis ENA profile) [46]
Anti ds-DNA	Present only occasionally in other autoimmune diseases besides SLE [50] Anti-ssDNA antibodies are non-specific and not used diagnostically [50] Prefer IIF test to confirm the positivity [53]
ACPA	Present also before disease onset. Specific for RA; they may be present even when the patient does not meet yet the criteria for RA [65]
RF	Common in primary Sjogren Syndrome (75-90%) and not specific of RA [78] Can be found in HCV, tuberculosis, syphilis [78] It may be present even in healthy subjects particularly >65 years old [79] Do not always correlate with RA disease activity [82]
Complement C3 and C4	Low not only in SLE but also, cryoglobulinemia, hereditary angioedema [86,87,123]
CPK	Increased in muscle injury with increased levels of AST as well [93] Levels correlate with severity of muscle injury [94]
Urine Analysis	Proteins/erythrocyte/leukocyte/casts in urine sediment, are signs of possible kidney involvement [114]
ESR	Less specific than CRP, increased in pregnancy, anemia, age [122]

Once a true positive titer is confirmed, the fluorescence pattern must be assessed. Approximately 29 distinct ANA patterns have been identified using IIF on HEp-2 cells, each associated with the potential presence of more specific autoantibodies, such as anti-double-stranded DNA antibodies (anti-dsDNA), anti-SSA/SSB, anti-centromere (ACA), among others [24,25]. Thus, the pattern alone may help to orient the differential diagnosis. For example, a homogeneous pattern is commonly associated with the presence of anti-dsDNA. In contrast, the speckled pattern, while frequent, is associated with a broader variety of autoantibodies and is therefore considered less diagnostically specific [26]. The nucleolar pattern should not be overlooked, as it may indicate a risk for systemic sclerosis (SSc) or idiopathic inflammatory myopathies [25]. Among ANA patterns, ACA are particularly relevant since they are highly specific for SSc, also frequently associated with increased risk of pulmonary arterial hypertension [27]. Particularly noteworthy is the dense fine-speckled (DFS) pattern, which is strongly associated with anti-DFS70 antibodies. This finding is of clinical interest because its presence, in the absence of other specific autoantibodies, can support the exclusion of a systemic autoimmune disease [28]. It is also important

to stress that there is no need to repeat ANA over the course of an already diagnosed CTDs, since ANA titer do not correlate with disease severity or progression [15,29]. The specialist could decide to re-test ANA in specific cases when there is a suspect of an overlap autoimmune disease or to check if the autoimmune serology has shifted over time.

As described before, IIF is the first-choice method recommended, but it accounts for several problematics since it is time consuming and it depends also on the reader experience leading to inter-observer variability [30]. It is to acknowledge that this method presents also a poor specificity and low predictive value. Due to these reasons, alternative methods for ANA detection have been developed mainly based on a solid-phase immune assay that includes ANA-targeted recombinant antigens such as ELISA and other typologies of assays for instance multiplex bead assays (MBA), chemiluminescence immunoassays (CLIA), fluoroenzyme immunoassays (FEIA), and addressable laser bead immunoassays (ALBIA). These techniques are primarily limited by the narrow range of purified or recombinant autoantigens they use, the absence of proper standardization, and the tendency to generate "false-negative" ANA results and do not exhibit a diagnostic sensitivity comparable to that of indirect immunofluorescence (IIF) [31,32]. Attempts to improve these assays have been made, and recent studies have reported improved sensitivity and higher specificity of ANA ELISA for CTDs screening. For example in [33], the sensitivity of ANA-IIF and ANA-ELISA for all CTDs was 63.3% vs 74.8% respectively, while the overall specificity of ANA-ELISA was higher than that of ANA-IIF (89.05% vs 86.72%). A metanalysis showed no significant difference between IIF and ELISA in terms of sensitivity and specificity; CLIA and IIF displayed comparable sensitivity, whereas FEIA performed significantly worse. However, both CLIA and FEIA offered greater specificity compared with IIF [34]. When interpreting autoimmune serological markers, clinicians should also consider the positive and negative predictive values (PPV, NPV) of each test, which vary depending on disease prevalence and clinical pretest probability. For example, the PPV of a positive ANA is low in individuals with nonspecific symptoms but increases substantially in patients with clear clinical features of CTDs. Based on all these premises the integration of IIF (the most sensitive method) with either CLIA or FEIA (the more specific assays) should result in superior diagnostic accuracy [35].

4. Anti-ENA and anti-dsDNA antibodies

Extractable Nuclear Antigens, commonly referred to by the acronym ENA, represent a second-line diagnostic test [36]. It is preferably performed following a positive ANA result by IIF, or when there is a strong clinical suspicion of a systemic autoimmune disease despite a negative ANA result [37]. The most common ENA antibodies are typically included in a standard panel (anti-ENA screening), which comprises Anti-SSA/Ro, SSB/La, RNP, Scl-70, Jo-1, and Sm antibodies, and is usually assessed using the ELISA technique [38]. Although the exact origin are not uniformly documented in literature, it is known that historically, several ENA specificities were named after the prototype sera of the first patients in whom these antibodies were identified, as originally described by early pioneers in the field such as Tan, Reichlin and Maddison [39] (for example Ro from 'Miss Rose', La from 'Miss Lane', Jo-1 from 'Mr Joan', and Sm from 'Miss Smith', a nurse who worked with Professor Tan).

The available technologies of second generation for detecting anti-ENA, as well as other autoantibodies, include not only ELISA (in its various forms such as direct, indirect, sandwich, and competitive ELISA), but also CLIA, which is similar to ELISA but utilizes chemical probes that emit light, line immunoassay (LIA), ALBIA, FEIA. Additional techniques include Western Blotting (or Immunoblotting) and its variants, such as Immuno Line-Blot (IB) and Immuno-Dot Blot (DB) [19,40,41]. As previously described for ANA, anti-ENA detection through different techniques presents some limitations. A positivity of ENA antibodies does not represent always the presence of an autoimmune disease [42]; also, the anti-ENA assays commercially available have variable sensitivity and specificity [15]. A study performed in 2022, explored eight different immunoassays (ELISA; CLIA, FEIA, LIA, multiplex flow immunoassay and two IB assays), to test antibodies against ENA (RNP, Sm, Ro60, La, Scl-70, Jo-1) across 60 patients with CTDs, 10 with RA and 10 healthy subjects finding differences among sensitivity while almost same specificity between the technologies [43]. On this base, in presence of ANA negativity, clinician should limit further testing for anti-ENA only in case of strong clinical suspicion [44].

The diagnostic workflow generally begins with either a positive ANA result or a strong clinical suspicion, followed by the anti-ENA screening (minimum standard panel) [45]. If the anti-ENA screening is negative or inconclusive, and depending on the ANA pattern and suspected organ involvement or the potential presence of rarer systemic autoimmune diseases, more specific panels may be requested at specialized centers [37]. These may include the expanded anti-ENA profile, the liver-specific anti-ENA profile, the scleroderma anti-ENA profile, or the myositis anti-ENA profile [46].

Anti-dsDNA are highly specific autoantibodies for SLE, discovered in 1957 [47–49]. If ANA are an entry criterion, anti-dsDNA positivity is the most important immunological criterion in the EULAR/ACR 2019 classification of SLE [22]. They are typically absent in drug-induced lupus, a less common form of SLE that should be suspected in patients presenting with SLE-like symptoms at an older age. Anti-dsDNA antibodies are only occasionally found positive in other autoimmune diseases [50,51]. It is crucial that in patients with suspected SLE, testing focuses on anti-dsDNA rather than anti-single-stranded DNA (anti-ssDNA), as the latter lacks diagnostic specificity and is not used in clinical diagnostics [52].

The analytical methods for anti-dsDNA detection include IIF, usually performed on *Crithidia luciliae* substrates (a flagellated parasite with kinetoplasts containing double-stranded DNA), where a titer of 1:10 is considered the minimum significant level [15]. Other methods include radioimmunoassay, regarded as a gold standard, though it has limited clinical application due to the use of radioactivity. ELISA can be used for quantitative assessment of anti-dsDNA antibodies; however, positive results should be confirmed by IIF, which offers greater specificity for SLE [53]. In practical terms, IIF is essential to verify the true presence of anti-dsDNA antibodies, whereas ELISA may be more suitable for monitoring changes in their levels over time. Indeed, while ANA and anti-ENA titers tend to remain stable, anti-dsDNA levels may fluctuate according to disease activity[54].

5. Do not overlook the thyroid

An important aspect to consider when requesting an extended autoantibody panel, particularly in cases of high clinical suspicion for autoimmune disease and in the presence of positive ANA, is the inclusion of thyroid autoantibodies, specifically anti-thyroid peroxidase and anti-thyroglobulin antibodies [55]. The positivity of these autoantibodies may occasionally serve as the sole explanation for ANA positivity, typically observed with a speckled or homogeneous ANA pattern [56] (Table 1). This scenario is not uncommon, given the relatively high prevalence of autoimmune thyroiditis, or Hashimoto's thyroiditis, in the general population [57].

6. Real-life clinical approach

In real-life clinical settings, when facing a patient, certain targeted questions can help orient the clinician toward a possible diagnosis of systemic autoimmune disease and assess whether it is appropriate to request further laboratory investigations, as for example in [58]. These questions also serve as a practical tool to be integrated with laboratory results, especially the detection of autoantibodies.

As outlined in the introduction, for the sake of brevity, this section presents a selection of guiding questions for the clinical suspicion of

systemic autoimmune diseases, commonly referred to as CTDs. It must be emphasized that these questions do not replace specialist evaluation and are only partial, as other autoimmune conditions, such as large- and small-vessel vasculitides, also require specific clinical consideration.

The most straightforward and essential questions are summarized, and the proposed diagnostic workflow is presented in Fig. 1. One particularly important item is the assessment of a family history of autoimmune disease, which should be interpreted broadly. The clinician should explore not only first-degree relatives but also extended family members, such as uncles and cousins, who may have been diagnosed with autoimmune conditions. Patients often deny such a history on initial questioning but may recall relevant information when prompted more specifically.

Investigation of Raynaud's phenomenon (RP) is another key element, as it may provide critical clues for ruling out rare conditions such as SSc. This topic would warrant a separate discussion, given the importance of obtaining detailed information, including age of onset, symmetry, triggers, and pattern of RP presentation. Most importantly, visual aids (e.g., photographs) should be shown to the patient to ensure accurate identification and to confirm that the reported symptoms truly correspond to RP [59]. It should be remembered that RP can also be idiopathic, especially when it arises at a very young age, or may be associated with other autoimmune diseases such as autoimmune thyroiditis, SLE, undifferentiated connective tissue disease, and others. So, based on these premises, it is crucial to distinguish primary from secondary RP. Nailfold videocapillaroscopy plays a key role in this differentiation: a normal capillaroscopic pattern strongly supports a diagnosis of primary RP, whereas abnormalities of scleroderma pattern such as giant capillaries, microhemorrhages, or avascular areas, are highly suggestive of secondary RP related to SSc spectrum disorders [60]. Details about the workflow of suspect/preclinical and very early diagnosis of SSc can be explored in [61].

Another potentially misleading symptom is oral aphthosis. It should only raise suspicion of autoimmunity when it meets criteria for *major* aphthosis that is characterized, in autoimmune and autoinflammatory conditions, by major ulcers in typical sites such as mucous membranes of the lips, buccal mucosa, tongue and soft palate. These lesions are larger than 1 cm, deep, and painful, with erythematous borders and a yellow-white necrotic base, often healing slowly and leaving scars [62]. In SLE different kinds of oral lesions can be found. The most typical are whitish plaque with erythema in the center and peripheric keratotic striae [63].

Questions regarding arthralgia and arthritis are complex but essential. It is important to remember that inflammatory joint manifestations are not limited to RA or psoriatic arthritis but may also be part of the clinical presentation of systemic autoimmune diseases such as Sjögren's syndrome, SLE, or idiopathic inflammatory myopathies.

7. Rheumatoid arthritis

7.1. *Anti-citrullinated protein antibodies*

Anti-citrullinated protein antibodies, commonly referred to by the acronym ACPA, are highly specific autoantibodies for RA [64]. They are often detectable even before the clinical onset of the disease and are extremely valuable for early diagnosis [65]. Furthermore, they are included in the 2010 ACR/EULAR classification criteria for RA [66].

The generation of these autoantibodies stems from the process of citrullination, during which the amino acid arginine is converted into citrulline by the enzyme peptidyl arginine deiminase (PAD) [65]. PAD2 is expressed by macrophages and dendritic cells and promotes citrullination in inflamed synovial tissue. PAD4 is primarily found in neutrophils and synoviocytes and plays a critical role in RA pathogenesis [67]. The citrullination process can become pathological when citrullinated proteins are mistakenly recognized as foreign by the immune system of genetically predisposed individuals. This recognition triggers an autoimmune response characterized by plasma cell activation, ACPA

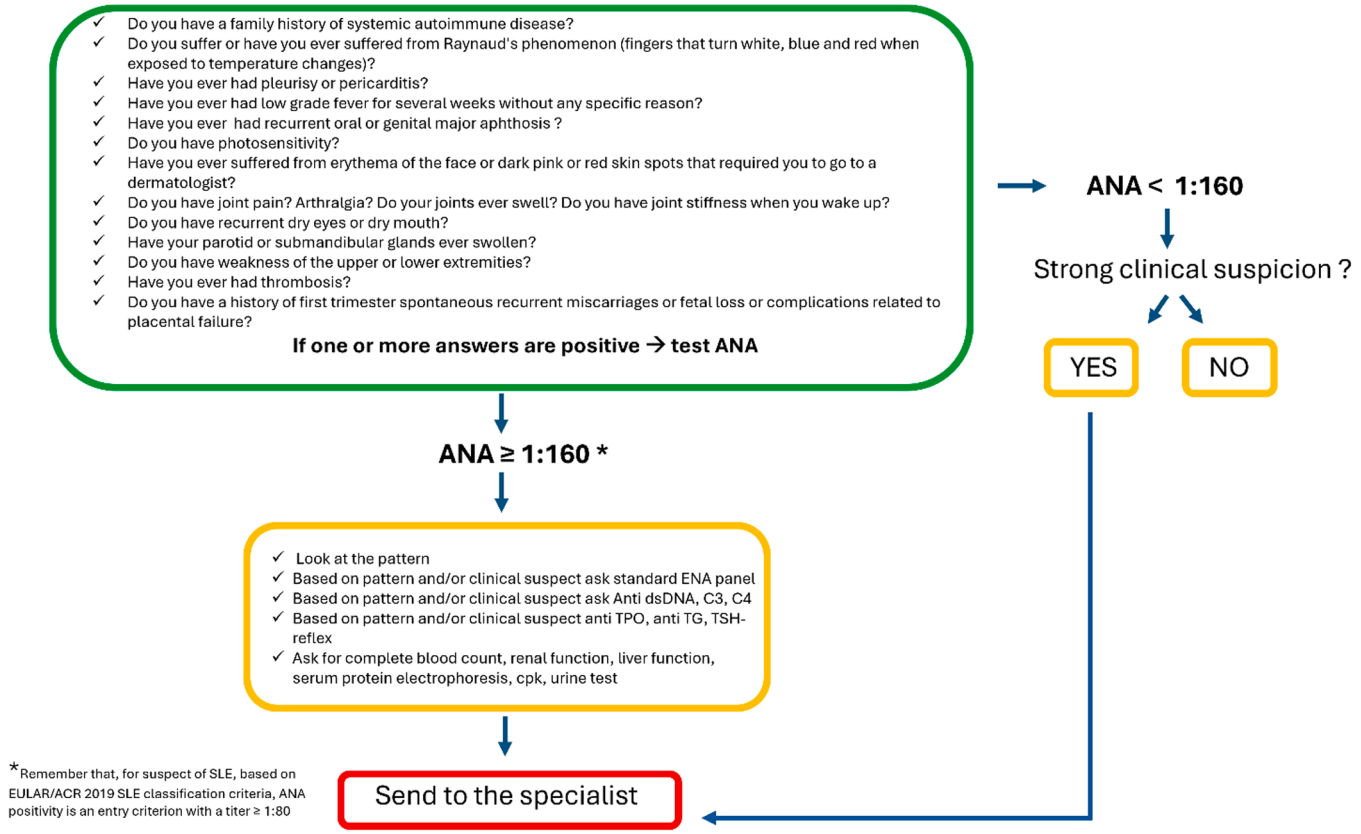

Fig. 1. Diagnostic workflow for clinical suspect of CTDs.

production, and the release of pro-inflammatory cytokines [68,69].

It is noteworthy that cigarette smoking is a recognized risk factor for developing seropositive RA. Smoking induces citrullination of proteins in the lungs, generating neoantigens that can be perceived as foreign by the immune system [70]. Smoking it is also associated with other autoantibodies production in RA [71].

7.2. Rheumatoid factor

Rheumatoid factor (RF) is an antibody directed against the Fc portion of IgG. RF and IgG together form immune complexes. The most commonly measured is RF IgM, although IgG, IgA (associated with disease severity) [72], and IgE RF isotypes also exist. RF detection methods include immunoassays (such as ELISA and CLIA) and agglutination tests (including latex agglutination and the Waaler-Rose test, the latter now rarely used due to its low specificity) [73–75]. Nephelometry and turbidimetry are also employed for RF quantification [76,77].

Elevated RF levels can be found not only in RA but also in other autoimmune diseases and chronic conditions, such as Sjögren's syndrome, as well as in infectious states (although it is not an acute-phase reactant in the strict sense) and even in healthy individuals, particularly those over the age of 60 (see Table 1) [78–80]. Therefore, a positive RF test alone is not diagnostic for RA.

Nonetheless, high RF titers in patients with RA are associated with more severe disease, including erosive joint damage and extra-articular manifestations such as rheumatoid nodules and vasculitis, and they are indicative of a poorer prognosis [81]. At the same time, low RF levels do not necessarily reflect disease remission, as RF titers do not always correlate with disease activity [82].

7.3. Real-life clinical approach

It can be challenging to clinically assess a patient presenting with new-onset arthritic symptoms. First, it is essential to investigate the symptoms carefully by asking highly specific questions: the precise location of the pain, whether it was accompanied by joint swelling (carefully distinguishing this from generalized edema, such as finger swelling due to circulatory issues), and whether morning stiffness was present and for how long (stiffness lasting more than 30 minutes, for example, supports the hypothesis of an inflammatory arthritis).

The clinician should inquire whether the joint pain is diffuse and migratory or consistently affects the same joints, whether the distribution is symmetrical, and whether there is a personal history of psoriasis or inflammatory bowel disease (IBD), or any recent infections. Also, involvement of the wrists and metacarpophalangeal joints is more suggestive of CTDs and RA, while pain affecting the shoulders or the lower back are much less specific and often point to alternative etiologies.

Arthritic manifestations may indicate RA, which may present positive or negative autoantibody profiles (seronegative RA). However, such symptoms could also be part of a different clinical context, such as CTDs (e.g., SLE, Sjögren's syndrome), reactive arthritis following recent infections, psoriatic arthritis, or arthritis associated with underlying IBD.

A definitive diagnosis requires specialist evaluation. Nevertheless, preliminary orientation can be achieved through correctly targeted screening questions and by referring the patient to a specialist with at least basic inflammatory markers already assessed C-reactive protein (CRP) and erythrocyte sedimentation rate (ESR). See Fig. 2 for the proposed diagnostic workflow.

8. Miscellaneous

8.1. C3 and C4

The complement fractions C3 and C4 are commonly used as markers of disease activity in SLE. C3 plays a central role in the activation of the complement cascade and is involved in both the classical and alternative

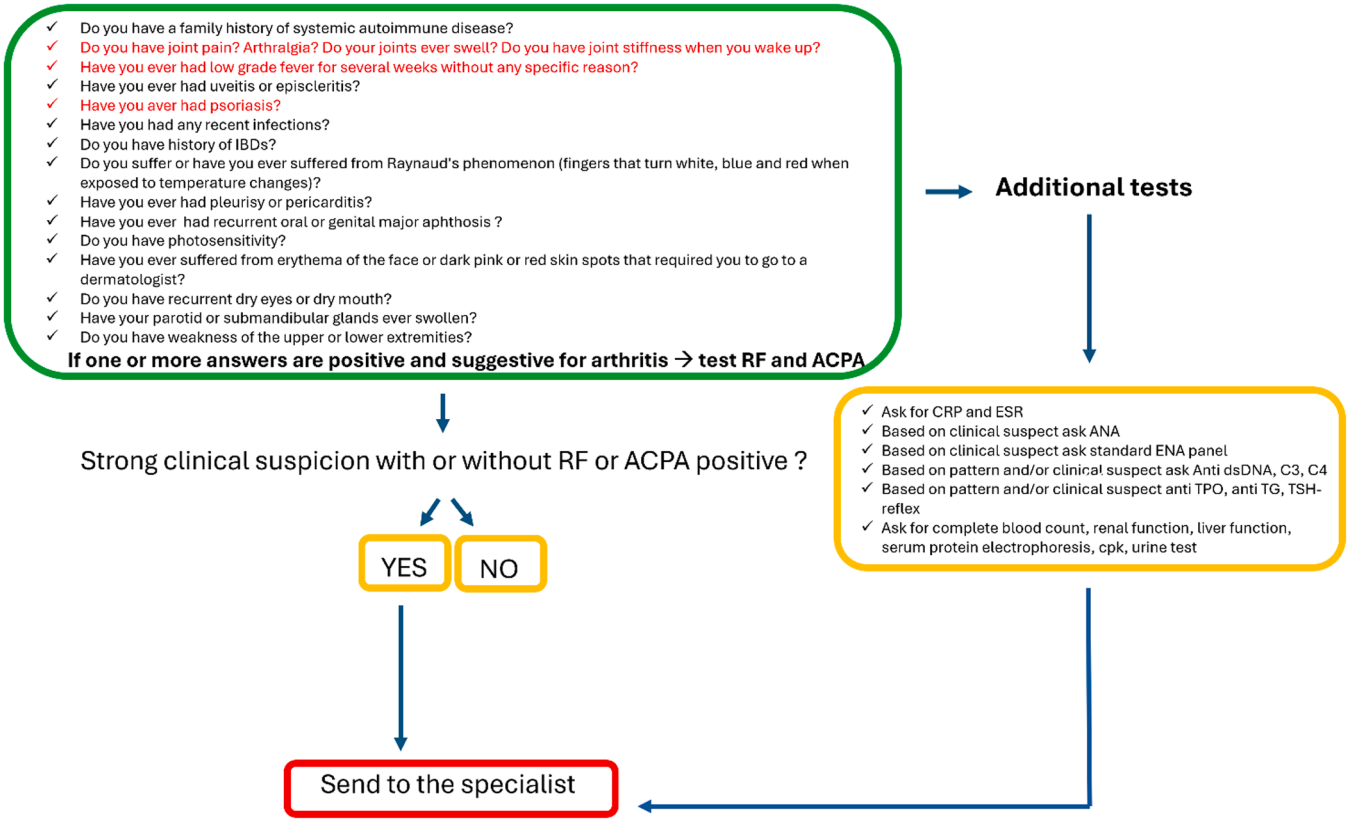

Fig. 2. Diagnostic workflow for clinical suspect of Rheumatoid Arthritis/Arthritic symptoms.

pathways, while C4 is part of the classical pathway and is activated by immune complexes [83]. Both C3 and C4 serve as indicators of complement consumption and may be decreased in patients with SLE, predicting disease activity and organ involvement [84]. In particular, serial measurements over time are more informative than single assessments since longitudinal trends of C3 and C4, provide a more reliable indication of SLE disease activity [85]. However, their reduction can also be observed in other conditions. For example, low C3 levels may be seen more specifically in lupus nephritis, whereas low C4 levels may be found in cryoglobulinemia or hereditary angioedema [86,87]. (see Table 1) Also, it is important to stress that clinicians should be aware of serologically active, clinically quiescent (SACQ) SLE, a state in which low complement levels or elevated anti-dsDNA antibodies persist despite the absence of clinical disease activity. Although in SACQ SLE about a 30-50% of patients could experience a flare of disease, misinterpreting these isolated serologic changes may lead to unnecessary therapies. Complement levels should therefore always be interpreted carefully within the overall clinical frame [88,89].

8.2. Creatine phosphokinase

Creatine phosphokinase (CPK) is an enzyme involved in energy metabolism, catalyzing the conversion of creatine and ATP into phosphocreatine, which is essential for muscle contraction and energy storage [90]. Several specific CPK isoenzymes exist: CPK-MM (skeletal muscle), where elevated levels indicate muscle injury; CPK-MB (heart muscle), where increased levels may suggest myocardial infarction or myocarditis; and CPK-BB (brain and smooth muscle), which may be elevated in stroke or malignancies [91,92].

In clinical practice, it is important to note that elevated CPK levels are often accompanied by increased aspartate aminotransferase (AST) levels [93] (Table 1). Both enzymes are released from muscle and other tissues following cellular injury, and the severity of muscle damage influences the extent of their elevation [94]. Clinically, it is not uncommon for patients to be referred for hepatology evaluation due to elevated transaminases; however, it is crucial to assess CPK levels, particularly when transaminase levels are significantly elevated without clear clinical evidence of liver disease, since this increment could be possibly related to inflammatory myopathy such polymyositis or dermatomyositis [95,96].

8.3. Serum protein electrophoresis

Without examining into the full analytical details of this test, which provides highly valuable information, it should be noted that patients presenting with polyclonal gammopathy (i.e., increased gamma globulin levels) should be evaluated for the possibility of underlying chronic infections or autoimmune diseases [97,98]. The gamma region reflects indeed the immunoglobulin production, including autoantibodies. It is also important to distinguish between a polyclonal and monoclonal gammopathies (MG): monoclonal spikes suggest a clonal plasma-cell proliferation, such as monoclonal gammopathy of undetermined significance (MGUS), and may require hematologic evaluation, whereas polyclonal hypergammaglobulinemia reflects a polyclonal B-cell activation pattern typically associated with chronic inflammation or autoimmune diseases [99]. MG finding is not uncommon in patients with autoimmune diseases and may warrant further evaluation depending on the pattern and clinical context (for example MG of rheumatologic significance such as cryoglobulinemia). To further explore this aspect, see [100].

8.4. Leukopenia and thrombocytopenia

Leukopenia, particularly neutropenia (low neutrophil count) or lymphopenia (low lymphocyte count), is commonly associated with autoimmune diseases [101]. Neutropenia is of particular clinical concern due to the increased risk of infection [102]. Lymphopenia is more typically seen in SLE and Sjögren's syndrome [103,104]. In clinical practice, leukopenia should be carefully assessed, and if suspicion arises, the patient should be referred for hematological evaluation, however, mild or stable leukopenia in a well-defined autoimmune context can often be monitored without immediate hematology referral. In the context of autoimmunity, several mechanisms may underlie leukopenia [105]: (1) Autoantibodies directed against white blood cells, leading to their destruction [106]. (2) Bone marrow suppression due to inflammation or autoantibodies targeting hematopoietic progenitor cells [107]. (3) Sequestration of white blood cells in organs such as the spleen (e.g., Felty's syndrome) [108]. (4) Concomitant infections associated with autoimmune diseases, such as viral infections (HCV, EBV, CMV), which can lead to transient leukopenia [109]. (5) Leukopenia secondary to immunosuppressive therapies, such as DMARDs (methotrexate, azathioprine, mycophenolate) or certain biologics (e.g., IL-6 inhibitors causing neutropenia) [110].

Similar to leukopenia, thrombocytopenia (platelet count $<150{,}000/mm^3$) may also occur in autoimmune diseases (especially CTDs), and the underlying causes largely overlap with those described for leukopenia [111]. However, certain conditions specifically associated with thrombocytopenia include immune thrombocytopenia (ITP), antiphospholipid syndrome (APS), and thrombotic thrombocytopenic purpura (TTP), which warrant further specific evaluation [112,113].

8.5. Urine analysis

Urine analysis is a cost-less and routine test particularly important to check kidney involvement in autoimmune diseases such as vasculitides or CTDs, especially in SLE [114]. Remember to check if protein or erythrocyte or leukocyte and/or casts are present in the sediment, since these are signs of possible kidney involvement. In this case, testing the 24 h urine proteins, and the analysis through the microscopic examination of fresh urine sediment, can rule out if the patients will need to perform a kidney biopsy. The urine protein/creatinine ratio can also be evaluated, as it provides a reliable estimate of protein excretion, quantifying renal involvement, particularly in conditions such as lupus nephritis [115]. Also, it is important to ask for the urine analysis each time a patient with history of CTDs or a young patient presents edema of the limbs or of the whole body.

8.6. CRP and ESR

CRP and ESR are inflammatory markers [116,117]. CRP is synthesized by the liver in response to acute inflammatory stimuli, whereas ESR measures the rate at which red blood cells settle at the bottom of a test tube, with higher rates indicating increased inflammation [118,119]. Both are fundamental markers in the evaluation of systemic autoimmune diseases, though they present several distinctive characteristics [120]: CRP rises rapidly, within 6-8 hours, while ESR increases more slowly, typically over 24-48 hours [121]. CRP is more specific for acute infections and inflammatory processes, whereas ESR is less specific and can be influenced by age, anemia, and physiological states such as pregnancy [122]. Thus, it is essential to consider the patient's comorbidities and physiological conditions (e.g., pregnancy) when interpreting ESR results (Table 1).

In general, isolated elevation of these markers, in the absence of clinical signs or additional diagnostic elements suggestive of autoimmune disease, should not automatically prompt referral for immunorheumatologic evaluation. Other potential causes must be excluded.

9. Conclusions

The diagnosis of systemic autoimmune diseases is complex and must be supported by a combination of clinical, diagnostic, and historical (anamnesis) findings. Laboratory testing provides valuable assistance

both in the initial suspicion phase and during patient follow-up. It is essential to always interpret serological markers in the context of the patient's comorbidities and to request these tests appropriately to minimize misinterpretations, paying close attention to potential pitfalls that may arise during the diagnostic process.

Declaration of competing interest

The authors declare that they have no conflict of interest.

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