Autoantibodies in connective tissue disease

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Abstract

Autoimmune connective tissue diseases are heterogeneous rheumatic diseases with the potential to affect multiple body systems. Autoantibodies are a characteristic feature of these diseases and are typically highly disease specific. In addition to aiding diagnosis, many autoantibodies have established associations with clinically important disease complications including internal organ involvement. In this chapter we review the autoantibodies relevant to autoimmune connective tissue diseases, excluding systemic lupus erythematosus, with particular reference to the associated clinical features and how identification of such an autoantibody may inform prognosis and clinical management. We also discuss the practicalities of testing for autoantibodies along with potential difficulties and pitfalls.

Key words

Autoantibodies

Connective tissue disease

Anti-nuclear autoantibody

Introduction

Autoantibodies can be considered a hallmark feature of the autoimmune connective tissue diseases, are typically highly disease specific and have been incorporated into disease classification criteria (1-5). Current understanding of the molecular mechanisms leading to autoantibody formation and their role in disease pathogenesis is limited. Given that known autoantibodies are directed against intracellular components, and are therefore not believed to interact physiologically with their antigenic target, they have generally been thought of as epiphenomena, related to immune dysregulation, rather than as key mediators of disease pathogenesis. This can be seen as at odds with the fact that such autoantibodies are highly disease specific and typically are mutually exclusive that is they do not occur alongside other autoantibodies. Similarly, it is intriguing that specific subgroups of antigenic targets are seen in different autoimmune diseases, for example ribonuclear proteins in systemic lupus erythematosus, the tRNA synthetases in inflammatory myopathies and centromere associated proteins in systemic sclerosis. Whilst it remains conceivable that autoantibodies may be able to penetrate cells and so have a primary role in disease (6, 7), the prevailing notion is that the target autoantigens themselves are more intimately linked with disease mechanisms and are presented to the immune system in an altered state, so driving an antigen driven immune response.

Regardless of their role in disease pathogenesis, autoantibodies are highly useful biomarkers able to facilitate diagnosis and inform prognosis. Autoantibodies can be used to identify those patients at higher risk of clinically important disease complications and therefore inform further investigation and monitoring. As yet, there is no evidence for differing treatment regimens based on autoantibody status however there is increasing awareness of autoantibodies associated with more severe disease and/or internal organ involvement which may benefit from a more aggressive treatment approach.

Here we discuss the connective tissue diseases in turn, along with relevant autoantibodies and how they may influence our clinical approach. Key autoantibodies and their associated connective tissue diseases are summarised in figure 1.

Autoantibody testing

Anti-nuclear antibodies

When investigating a patient with suspected connective tissue disease for autoantibodies the first step is often to request testing for anti-nuclear autoantibodies (ANA). There are a number of different platforms for detecting ANA including blotting, ELISA or immunofluorescence. Immunofluorescence is considered the standard method for screening, although other assays are normally needed to identify the autoantibody specificity. The sensitivity of immunofluorescence has been increased by using cultured human cell lines rather than tissue sections. The majority of laboratories use HEp-2 cell substrates, and this is the authors' own preference. ANA detection by blotting or ELISA can miss non-standard autoantibodies and immunofluorescence has the advantage of potentially detecting novel or unknown autoantibodies and providing additional information by way of the immunofluorescence pattern. Immunofluorescence pattern in the clinical context can then inform appropriate further investigation to identify autoantibody specificity and further refine the clinical significance. Anti-nuclear antibodies do occur in healthy individuals, particularly at lower dilutions, and immunofluorescence pattern can provide an important clue to potential clinical significance of a positive finding. It is interesting that the cellular location of autoantigens, and hence staining patterns can be disease associated, for example SSc autoantigens frequently produce centromere or nucleolar staining patterns while myositis autoantigens are most commonly located in the cytoplasm. An anti-nucleolar staining pattern in a patient with Raynaud's phenomenon, should be seen as suspicious for SSc even in the absence of an identifiable SSc associated autoantibody.

The International Consensus on ANA Patterns (ICAP) initiative has recently reached a consensus on the nomenclature of HEp-2 IIFA patterns and has defined 29 distinct patterns (8). Key immunofluorescence patterns associated with different connective tissue diseases are described in table 1. It is important to remember that cytoplasmic staining patterns (as seen with many myositis associated autoantibodies) may not be reported by all laboratories. Anti-Ro patients are not always ANA positive and ENA testing in a patient with a negative ANA should be considered where there is clinical suspicion.

Identifying target autoantigens

There are again a variety of laboratory methods to detect extractable nuclear antigens (ENA) and other target autoantigens. Techniques such as double immunodiffusion or counter current immunoelectrophoresis are now rarely used in favour of modern commercial assays such as line blot and other multiplex immunoassays. Multiplex assays are commercially available to detect a spectrum of autoantibodies associated with a particular connective tissue disease for example as a myositis or SSc panel. Like all laboratory tests false positive and negative results occur and findings should always be interpreted in the clinical context. An understanding of the anticipated immunofluorescence pattern can also be useful in identifying potential false positive results. An inconsistent immunofluorescence pattern or the apparent identification of multiple autoantibody specificities in a patient with myositis or SSc should raise alarm bells for a false positive result.

It should also be remembered that the connective tissue diseases have many overlapping clinical features and testing via a disease specific autoantibody panel may inadvertently lead to a patient being labelled autoantibody negative if their autoantibody is more commonly associated with another connective tissue disease. A recent study of over 100 SSc patients who were ANA negative identified autoantibodies targeting a known autoantigen in 29% and unknown bands on immunoprecipitation, suggesting as yet uncharacterised autoantibodies, in a further 36% (9).

Interestingly, known autoantibodies identified in this study included anti-synthetase autoantibodies in over 5% of patients, in addition to the rarer, more recently described SSc autoantibody, anti-EIF2B (9). Consideration should be given to testing for anti-synthetase autoantibodies (most typically included in a myositis panel) in an autoantibody negative patient presenting with signs consistent with SSc. It may also be worthwhile to consider specialist autoantibody profiling, using techniques such as immunoprecipitation, in select patients where an autoantibody has not been identified. Rarer and more recently described autoantibodies are not generally included in standard testing panels. Alternatively, the treating physician may have a particular concern regarding false positive or false negative results from previous testing and confirmation would be useful. If the presence of an autoantibody could affect clinical management, for example in the UK NICE recommend the use of rituximab in myositis only in patients who have a myositis-related autoantibody, the authors would recommend that extended spectrum autoantibody testing is strongly considered.

Myositis

An autoantibody can be identified in approximately 60% of patients with myositis spectrum disease (10, 11). 'Myositis-specific autoantibodies' are considered specific to the idiopathic inflammatory myopathies and are not found in other connective tissue diseases, although this is arguably a misnomer as not all patients have muscle involvement. 'Myositis-associated autoantibodies' in contrast can be identified in patients with myositis, overlap disease and other connective tissue diseases. While myositis-specific autoantibodies are mutually exclusive and occur together only extremely rarely, myositis-associated autoantibodies may be found in conjunction with a myositis-specific autoantibody or another myositis-associated autoantibody (10, 11).

The term myositis describes a heterogeneous groups of diseasea and division into the traditional subgroups of polymyositis, dermatomyositis and inclusion body myositis inadequately describes all of the variation seen in both clinical features and disease outcome. Patients with what was

previously described as polymyositis usually fall into the now preferred categories of anti-synthetase syndrome or immune-mediated necrotizing myopathy, but we argue that autoantibody status provides a greater level of phenotypic differentiation. Different myositis disease phenotypes and their relationship with autoantibody status is described below.

Anti-synthetase syndrome:

The anti-synthetase syndrome is a well described clinical syndrome consisting of myositis, interstitial lung disease (ILD), non-erosive arthritis, Raynaud's phenomenon, fever, and characteristic skin changes termed 'mechanics' hands' (12). Patients have autoantibodies directed against tRNA synthetases, a family of cytoplasmic enzymes responsible for catalysing the binding of amino acids to their cognate tRNAs (12). There are 20 different tRNA synthetases and autoantibodies targeting eight have thus far been described. Anti-Jo-1, targeting histidyl tRNA synthetase, is the most common autoantibody in adults with myositis and is found in 15-30% of patients (10, 12). The remaining anti-tRNA synthetases; anti-PL7 (threonyl), anti-PL12 (alanyl), anti-OJ (isoleucyl), anti-KS (asparginyl), anti-EJ (glycyl), anti-Zo (phenylalanyl) and anti-Ha (tyrosyl) are rarer, collectively occurring in 10-20% of cases (10, 12). The anti-synthetase syndrome is generally considered to be a single clinical syndrome but incomplete versions are frequently seen and not all patients have muscle involvement. Furthermore, there are established differences between the clinical associations of the different anti-synthetase autoantibodies and while muscle disease is common in patients with anti-Jo1, anti-PL-7 or anti-EJ, those with anti-PL-12, anti-KS or anti-OJ in contrast often have lung dominant disease (13-19). We would recommend considering anti-synthetase syndrome and testing for anti-synthetase autoantibodies in patients presenting with ILD, even in the absence of muscle involvement or characteristic dermatomyositis skin changes. ILD is a major cause of mortality in myositis and occurs in up to 90% of adults with anti-synthetase syndrome (20, 21). Survival is worse for patients with non-Jo-1 anti-synthetase autoantibodies which may reflect that these patients are more likely to present with incomplete versions of the syndrome, without muscle involvement and/or additional challenges in autoantibody identification and diagnosis (13).

Dermatomyositis:

Anti-Mi2 is generally considered the archetypal dermatomyositis autoantibody but is relatively rare and is found in only around 5% of affected adults (10). Affected patients typically present with significant skin and muscle involvement. While these patients generally respond well to standard treatment, and are considered to have a good prognosis, a recent very large study of European adults with myositis has demonstrated an association between anti-Mi2 and malignancy (10).

Anti-NXP2 and anti-TIF1γ which can be found in 2 and 7% of European adult myositis patients respectively have also been linked with malignancy (10, 22-24). Data suggests that patients with anti-NXP2 autoantibodies are more likely to have severe muscle disease involvement and possibly calcinosis (11, 24). In contrast, anti-TIF1γ may have milder muscle involvement and amyopathic dermatomyositis, that is the cutaneous features of dermatomyositis occurring in the absence of muscle involvement, has been described (25, 26). Patients with anti-MDA5 autoantibodies typically have characteristic dermatomyositis cutaneous disease with minimal or no muscle involvement. Deep, punched out ulcers can be seen, including ulcerated gottron's papules which may reflect a significant underlying vasculopathy(27, 28). Anti-MDA5 is more common in East Asian cohorts and is particularly notable for its association with ILD, rapidly progressive ILD and high associated mortality (10, 29-31). The presence of skin ulceration has been associated with a higher risk of ILD in patients with anti-MDA5 (32).

Immune-mediated necrotising myopathy:

This recently described subgroup of myositis spectrum disease is strongly associated with anti-SRP and anti-HMGCR autoantibodies. Patients typically present with profound muscle weakness and significantly elevated creatinine kinase levels. Muscle biopsy is characteristic with marked myofibre necrosis and minimal or no inflammatory infiltrate. Despite the lack of muscle inflammation on biopsy, patients usually do respond to immunosuppression, although this group may be refractory to standard treatment regimens and require a more aggressive approach.

Anti-HMGCR autoantibodies target 3-hydroxy-3-methylglutaryl-coenzyme A reductase, an important enzyme in cholesterol biosynthesis that is upregulated by statins. Intriguingly, anti-HMGCR positive myositis is associated with statin use and 40-60% of affected patients have been exposed to statins (33, 34). Anti-HMGCR patients with a history of statin exposure are reported to respond well to statin withdrawal and standard immunosuppressive treatment. Statin naïve patients in contrast may be refractory to usual therapy (33, 35).

Inclusion body myositis:

Inclusion body myositis (IBM) is clinically distinct from the other myositis disease phenotypes: It is more common in men, leads to a different pattern of muscle weakness, notably including finger flexors and knee extensors, and is insidious in onset. It does not respond to immunosuppressive treatment. These differences have prompted questions as to whether IBM is in fact a degenerative disease rather than an inflammatory muscle disease. The discovery of autoantibodies directed against cytosolic 5'-Nucleotidase 1A (cN1A) in patients with inclusion body myositis provides strong support that it is indeed an autoimmune disease process (36, 37). Anti-cN1A have been reported in 30-50% of patients with sporadic inclusion body myositis but do not appear to be specific and have also been identified in other systemic autoimmune rheumatic diseases, including Sjogren's syndrome and Systemic Lupus Erythematosus (SLE) (38). Anti-cN1A autoantibodies in patients with IBM are associated with a higher mortality risk, independent of age, gender, comorbidities and the presence of dysphagia (39).

Systemic Sclerosis

Systemic sclerosis (SSc) is characterized by immune mediated microvascular dysfunction and tissue fibrosis. Autoantibodies can be identified in >95% of all patients and provide detailed information on prognosis and clinical phenotype. Furthermore, the presence of a SSc autoantibody in a patient with Raynaud's phenomenon should be considered highly predictive for the later development of SSc.

SSc is typically clinically subdivided into limited cutaneous and diffuse cutaneous forms. Sclerodermatous skin changes limited to distal to the elbows and knees with or without facial involvement is considered limited cutaneous (lcSSc) whereas in diffuse cutaneous SSc (dcSSc) skin changes are more extensive. While the extent of skin involvement is the traditional method for subclassifying patients with SSc, as with myositis described above, autoantibody status can provide more detailed information and, could form part of a more modern approach to predicting phenotypes (40).

Autoantibodies associated with limited cutaneous SSc:

Autoantibodies directed against centromeric proteins (ACA) are the most common autoantibodies in patients with SSc and are found in 20-38% (41). ACAs produce a distinctive ANA pattern that does not require further confirmatory testing. Raynaud's phenomenon is nearly always the first symptom and can preceed the development of other clinical features by several years. Patients with ACA nearly always have limited skin changes, the disease typically progresses slowly and negative associations with visceral involvement confer a better prognosis. Pulmonary arterial hypertension is a well-recognised late complication and affected patients should be actively monitored for this (41).

Other autoantibodies more commonly associated with lSSc are anti-Th/To (1-13%) and anti-U11/12RNP (3%) (41). While phenotypic data is limited for these rarer autoantibodies, both are associated with interstitial lung disease. Anti-Th/To are also associated with a higher frequency of pulmonary hypertension, SSc renal crisis and a reduced survival (42). Patients with anti-PmScl autoantibodies present with features of both myositis and SSc. This group can present very similarly to the anti-synthetase syndrome with Raynaud's phenomenon, inflammatory joint disease, puffy swollen fingers and limited cutaneous SSc.

Autoantibodies associated with diffuse cutaneous SSc:

Anti-topoisomerase autoantibodies (previously known as anti-Scl70) are the most common autoantibody in those with dSSc and are found in 15-42% of all patients. Anti-topoisomerase are

associated with an increased risk of ILD, digital ulceration, cardiac involvement and a poor prognosis (41). Antibodies directed against RNA polymerases are also more typically associated with dSSc skin change: Anti-RNAPol I and III almost always co-exist and are highly specific for SSc. In contrast, anti-RNAPol II can be found in SSc but also other autoimmune connective disease diseases and overlap syndromes. Anti-RNA Pol I/III are found in 5-31% of SSc patients, they are associated with a higher risk of SSc renal crisis. Importantly, anti-RNA pol III is also associated with an increased risk of SSc associated malignancy particularly within 3 years of SSc onset (43). While routine cancer screening in such patients does not yet have an evidence base, we suggest a degree of suspicion should be maintained and further investigation arranged if clinically appropriate. Anti-U3RNP (also known as anti-fibrillarin) has been found in 1-3.5% of SSc patients. A distinctive 'clumpy nucleolar' ANA pattern can be seen but autoantibody specificity should be confirmed by further testing. Anti-U3RNP is more common in patients of Native American and African American ethnicity, it is associated with high skin scores, organ involvement and an increased mortality (41, 44-46). Recently described, anti-EIF2B autoantibodies have been found in just 1% of patients with SSc. Phenotype data is limited but associations with dcSSc and interstitial lung disease are reported (9, 47).

Sjogren's Syndrome

Sjogren's syndrome (SS) can occur alone (primary) or as part of a connective tissue disease such as rheumatoid arthritis or SLE. In mild disease, patients may complain of sicca symptoms with fatigue, myalgia, and mild cognitive dysfunction. More severe disease may cause florid salivary gland enlargement, adenopathy, low complement levels, the presence of cryoglobulins, hypergammaglobulinaemia, extra-glandular features and a predisposition to Non-Hodgkin's lymphoma (NHL). Extra-glandular manifestations include neuropathies, nephropathies, interstitial pneumonitis, hematologic abnormalities and lymphoproliferative changes.

SS is associated with positive ANA by immunofluorescence and with rheumatoid factor (RF), although the prevalence of both of these findings increases with age so much so that up to 1 in 5 patients over the age of 80 may be positive for these autoantibodies (48). Autoantibodies, namely to Ro and La are not diagnostic of the disease without clinical correlation as they can be falsely positive. This is particularly true of weak-positive anti-La antibodies when tested for by solid-phase assays (49). The most recent ACR/EULAR classification criteria of 2016 therefore require anti-Ro positivity or a positive labial salivary gland biopsy showing lymphocytic sialadenitis in addition to objective findings of either dry eye or reduced salivary flow (3).

Anti-Ro antibodies actually consist of two distinct antibodies with specificity for the Ro52 kD and Ro60 kD proteins. Patients testing positive for anti-Ro antibodies will therefore have a combination of one or both of these antibodies. Ro52 is usually located within the nucleus and whereas Ro60 is in the cytoplasm. Patients with specificity to either Ro52 and/or Ro60 may be ANA-negative using the HEp-2 substrate due to the fact that Ro52 may not be expressed by this cell line and Ro60 is mainly found within cytoplasm and cytoplasmic staining may not be reported. Although anti-Ro antibodies form the major basis of disease classification in SS, they can be present in other rheumatic diseases including SLE (50), anti-synthetase syndrome (51), rheumatoid arthritis (52), SSc, mixed connective tissue disease (MCTD), undifferentiated CTD (UCTD) and non-rheumatic autoimmune diseases such as primary biliary cholangitis (PBC). SS patients positive for anti-Ro52 also have a more aggressive phenotype (53) as is found in other autoimmune diseases such as MCTD (54). Compared to anti-Ro, anti-La antibodies are specific to SS and SLE (55) although some authors suggest that La-positivity alone does not produce the SS phenotype (49). Unlike Ro, the La protein shuttles between the nucleus and cytoplasm and immunofluorence using HEp-2 cells will provide a positive ANA test which is usually speckled. A small proportion of SS patients may also have an anti-centromeric pattern on immunofluorescence and show signs of sclerodactyly with Raynaud's phenomenon. These patients have more pronounced exocrine gland dysfunction compared to ACA-negative SS patients (56). However, a comparison of ACA between SS and SSc differ in their centromere protein

(CENP) targets: The ACA in SS target only CENP-C whereas the majority of ACA-positive SSc patients have specificities towards both CENP-B and CENP-C (57). There are of course many autoantibodies which can be detected in SS serum which but which are currently limited to the realm of research (reviewed in (58)). Of most interest, autoantibodies to salivary protein-1 (SP-1), carbonic anhydrase 6 (CA6) and parotid secretory protein (PSP) were positive in 76% of early SS patients compared to only 31% for anti-Ro and anti-La. These novel autoantibodies therefore may prove to be of use when diagnosing early disease and in those who lack anti-Ro and/or anti-La (59).

Anti-Ro and anti-La can also cause autoimmunity by passive transfer. The most striking example of this is neonatal lupus syndrome which occurs when mothers positive for anti-Ro and/or anti-La passively transfer these autoantibodies onto their unborn babies causing a transient lupus-like rash with deranged hepatic and haematologic parameters. Of most concern is the development of congenital heart block which can occur in 1 – 2 % of mothers with anti-Ro +/- anti-La positivity. This can increase to 15 % of pregnancies if a mother has already given birth to a baby with neonatal lupus syndrome (60). Furthermore, mothers of babies with neonatal lupus or congenital heart block may or may not have any signs or symptoms of a connective tissue disease and must therefore be tested for autoantibodies. Ex vivo experiments have shown that anti-Ro52, anti-Ro60 and anti-La all have the ability to recognise their cognate antigens in foetal heart tissue (61). Seropositive mothers should then go on to have increased frequency of screening including foetal echocardiogram, for the development of congenital heart block during their pregnancies. Furthermore, healthy mothers with no prior evidence of a connective tissue disease may go on to develop SS if they are subsequently found to have anti-Ro/La antibodies after having had a baby with neonatal lupus syndrome (62).

Antiphospholipid syndrome

Antiphospholipid syndrome (APS) is an autoimmune condition causing a hypercoagulable stable which leads to a range of clinical presentations including venous and arterial thromboemboli,

obstetric complications such as pregnancy loss and stroke-like symptoms (63). Catastrophic antiphospholipid syndrome (CAPS) causes systemic micro-emboli within organs and can rapidly lead to multi-organ failure (64). Rather than immunosuppression to suppress autoantibody generation, the mainstay of treatment is anticoagulation. APS can occur alone as a primary disease or as part of another connective tissue disease with SLE being the most common coexisting condition.

Autoantibodies are targeted against a host of autoantigens which can be a combination of components of cell membrane phospholipids (so-called antiphospholipid antibodies) or of the clotting cascade itself which can then lead to complement activation, further exacerbating the hypercoagulable state (reviewed in (65)).

Anticardiolipin antibodies (aCL) are targeted against cardiolipin, a phospholipid found almost exclusively on the mitochondrial membrane and bacterial cell walls (66), therefore making them a type of anti-mitochondrial antibody. The presence of aCL doubles the risk of recurrence of thromboembolism compared to those without the antibody (67), particularly after cessation of anticoagulation, and therefore patients benefit from long-term anticoagulation.

Anti-β2 glycoprotein-I antibodies (aβ2GPI) target the multifunctional plasma protein β2 glycoprotein-I. This protein binds cardiolipin which in turn causes a conformational change in its structure leading to further phospholipid binding and modifies coagulation (as reviewed by (68)). The complex of β2 glycoprotein-I with aβ2GPI appears to upregulate tissue factor and cause activation of endothelial cells, monocytes, neutrophils, fibroblasts and trophoblasts and leads to a more pro-coagulable state (68). Moreover, it appears that only antibodies to domain 1 of β2 glycoprotein-I produce increase the risk of thrombosis by an odds ratio of 3.5 (69).

Lupus anticoagulant (LAC) is the term used for a number of indirectly measured autoantibodies directed against membrane phospholipids. Paradoxically, *in vitro* it causes prolongation of the clotting time due to phospholipid binding which decreases the binding capability of prothrombinase complex and therefore delays the clotting cascade. Unlike aCL and aβ2GPI which can be measured

directly, it is measured by diluting test plasma with normal pooled plasma and seeing no change to the APTT ratio which infers that rather than a clotting factor deficiency in the sample there must be an inhibitory antibody.

Anti-prothrombin antibodies (aPT) and anti-phosphatidylserine/prothrombin complex antibodies (aPS/PT) are not included in the diagnostic classification criteria for APS but they are associated with increased venous and thrombosis risk, with a systematic review finding that the highest risk of thrombosis is with the presence of aPS/PT with an OR of 5.11 (70). Furthermore, other non-conventional antiphospholipid antibodies can be detected in APS sera, highlighting that there are multiple pathways implicated in the disease process, and non-supervised hierarchical clustering found a strong association between aPS/PT and LAC (71). This finding may be particularly pertinent to seronegative APS whereby classical autoantibodies are negative, particularly when LAC cannot be measured due to concurrent anticoagulation, and the strong correlation of aPS/PT with LAC may make solid-phase testing of this antibody more widespread in the future (72).

Although not thought to have an inflammatory component, it is becoming increasingly evident that complement activation is needed for the formation of thromboses in APS from work in mice deficient in certain complement proteins (65). Moreover, aβ2GPI co-localises with complement proteins in the arterial walls of APS patients with arterial thromboses who also have increased circulating C5A and C5b-9 (73) with C5 being shown to be an important component for thrombosis formation (74). Furthermore, the monoclonal anti-C5 therapy eculizumab has been successfully used to treat CAPS in a number of cases (75, 76). These reports suggest that complement activation plays a pivotal role in the progressive micro-angiothrombotic nature of CAPS. Current EULAR guidelines reflect this and suggest that eculizumab may be considered for the treatment of refractory CAPS (77).

Unprovoked thromboembolism in younger patients (< 50) or recurrent pregnancy loss should prompt autoantibody testing. For stratification into low, medium or high-risk groups, testing should

be done on at least 2 occasions at least 12 weeks apart (77). Classification criteria were updated in 2006 from the preliminary Sapporo criteria to include at least one clinical and one laboratory feature (5). The laboratory features include:

- The presence of LAC detected by a standardised detection assay as set out by the International Society on Thrombosis and Haemostasis (78), on 2 or more occasions at least 12 weeks apart
- The presence of aCL (IgG and/or IgM isotype) present in medium or high titres by a standardised ELISA, on 2 or more occasions at least 12 weeks apart
- The presence of aβ2GPI (IgG and/or IgM isotype) present in titres greater than the 99th percentile by a standardised ELISA, on 2 or more occasions at least 12 weeks apart

Testing LAC, aCL and aβ2GPI is also able to stratify patients into risk groups depending on their double- or triple-positivity status. For instance, LAC has been found to have the highest thromboembolic risk (odds ratio 4.4) compared to aβ2GPI and aCL, but being triple-positive causes the odds ratio to increase to 33.3 (79). In fact, having APS and being triple-positive also gives the worst obstetric outcomes despite anticoagulation (80). Seropositive status also has implications for treatment options. Recently, Pengo et al. showed that warfarin is superior to rivaroxaban, a direct [anti Xa] oral anticoagulant, in preventing recurrent thromboses in triple-positive APS patients (81).

Overlap disease

Overlap disease is used to describe those patients who fulfil classification criteria for more than one autoimmune connective tissue disease and is often taken to include MCTD. Anti-PmScl, anti-Ku, anti-NOR90, anti-U1RNP are typically found in patients with overlap disease. Patients with anti-PmScl classically have features of both myositis and SSc, are at risk of interstitial lung disease. Clinical presentation can be very similar to the anti-synthetase syndrome with ILD, mechanics hands, Raynaud's phenomenon and arthralgia. Anti-Ro52 can occur in isolation or in conjunction with another

connective tissue disease associated autoantibody. Anti-Ro52 commonly occurs alongside other anti-synthetase autoantibodies and has been associated with more severe interstitial lung disease (82, 83).

Mixed connective tissue disease, characterised in 1972 due to the overlap of CTD symptoms it can produce including those of SLE, myositis, dermatomyositis, RA, ILD, and SSc (78). It is now recognised as a distinct entity in its own right and is defined by the presence high titres of anti-U1 RNP antibodies (84, 85).

The compartmentalization of functions within eukaryotic cells has necessitated proteins and RNA to associate together into large ribonucleoprotein (RNP) complexes including nucleosomes (86) and spliceosomes (87). Spliceosomal components consist of short RNA fragments from 80 to 350 nucleotides in length, labelled U1 - U6 which complex with many different proteins and are known as small nuclear RNP (snRNP). Whereas the anti-Sm antibody found in SLE targets the common core of the RNA-protein particle complexes, anti-RNP antibodies found in MCTD specifically precipitate a 70K protein which uniquely associates with U1 RNA to form the U1 snRNP (88). Furthermore, U1 RNP is able to active the innate immune system via toll-like receptors (TLRs) thereby acting as a bridge between innate and adaptive immunity (89), which is likely to lead to autoimmunity, especially given that patients with MCTD have CD4+ T cells which have a restricted repertoire reactive against the 70K U1 RNP particle (90).

There are many overlapping features of SLE and MCTD, including autoantibodies, which has raised the suspicion that they may actually be part of a spectrum of disease, but given that SLE patients primarily exhibit the IgM isotype of anti-U1 RNP whereas MCTD patients are much more likely to develop class-switched IgG autoantibodies against U1 RNP suggests that the two diseases are in fact separate entities (91). Furthermore, other autoantibodies prevalent in other CTD have been shown to increase the risk of ILD in MCTD, for instance positivity for anti-Ro52 leads to increased risk of ILD (54).

The 1989 Alarcon-Segovia criteria for classification of MCTD includes a high titre U1 RNP test (>1:1600) plus three out of the five clinical features of hand oedema, synovitis, myositis, Raynaud's phenomenon and sclerodactyly, with a sensitivity and specificity over 90% and 98%, respectively (92). It is widely accepted that MCTD has a lower prevalence of serious renal or central nervous system disease compared to SLE and is responsive to corticosteroids (87). Although IgG U1 RNP autoreactivity is the hallmark of MCTD there are multiple other class-switched autoantibodies present in the disease directed against post-translationally modified U1 RNP (93), hnRNP A2, anti-Ro, and other non-U1 RNPs (reviewed in (94)). The autoantigen epitopes in MCTD and indeed other connective tissue diseases include an RNA-binding motif which is the basis of epitope spreading to other antigens with RNA-binding motifs, in these diseases (95). Interestingly, the development of the clinical phenotype of CTD may be dependent on the genotype of a patient and in particular their HLA repertoire (96). MCTD has a major association with DRB1*04:01 and HLA-B*08 when compared to SLE, SSc and PM/DM (97). Therefore, in the context of autoimmunity, the major histocompatibility complex on chromosome 6 may be implicated in influencing the direction of epitope spreading amongst autoantigens by the affinity of the peptide-binding grooves of each individual HLA molecule with known autoantigens.

Practice points.

- Autoantibodies are highly specific and are selectively expressed within connective tissue disease subsets
- Autoantibodies facilitate diagnosis and provide important information on prognosis
- Results of autoantibody testing should always be considered in the clinical context. If available, further testing should be considered where false positive or false negative results are suspected

- Consider tailoring further investigation and monitoring in a patient with an autoantibody associated with internal organ involvement or malignancy
- Consider a more aggressive treatment approach in a patient with an autoantibody associated with severe disease or internal organ involvement

Research agenda.

- A greater understanding of the role of autoantibodies in disease pathogenesis may identify novel therapeutic targets
- While it is clear autoantibodies influence prognosis and disease outcome, further work is needed to provide clear guidance on how autoantibody status should influence further investigation and our choice of treatment

Summary

Autoantibodies are a characteristic feature of the autoimmune connective tissue diseases, they are highly disease specific and define phenotypically distinct disease subgroups. Autoantibodies are currently clinically useful biomarkers to facilitate diagnosis and inform prognosis but more detailed guidance is needed on how autoantibody status should influence our approach to further investigation and treatment.

There are a number of methods for detecting autoantibodies each with their own inherent limitations. Physicians should have an understanding of these in order to best interpret results in the clinical context. Extended spectrum autoantibody testing may be beneficial in some circumstances.

The relationship between autoantibodies and disease pathogenesis remains largely unknown but recent studies challenge our understanding of the immune response and suggest mechanisms whereby autoantibodies may not simply be epiphenomena but could play a key role in disease

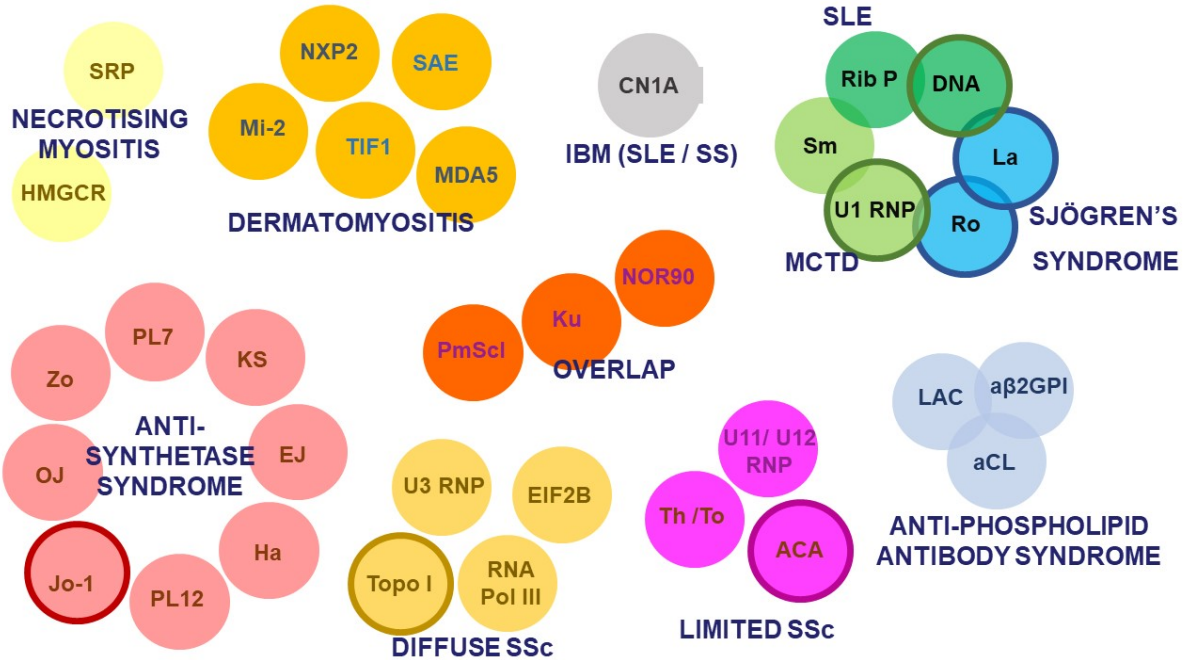
pathogenesis. Future research in this area could lead to novel therapeutic targets and a more personalised approach to treatment.

Table 1. Guidance on Interpreting Immunofluorescence Patterns

Nuclear	
Homogeneous	Pattern seen in patients with SLE where it is associated with anti-dsDNA autoantibodies and anti-histone autoantibodies. Also the most prevalent ANA pattern in patients with juvenile idiopathic arthritis and chronic autoimmune hepatitis.
Centromere	Pathognomonic of anti- CENP proteins and strongly associated with limited cutaneous SSc. Care should be taken to distinguish the classic pattern from that caused autoantibodies to CENP F. Anti-CENPF produces a similar staining pattern but interphase nuclei are not stained. These autoantibodies are rarely seen in patients with SSc but are strongly associated with malignancy (91). In contrast to ACA in scleroderma which recent evidence suggests areassociated with a decreased risk of cancer (98).
Nucleolar	Frequently seen in patients with SSc and should always raise suspicion of a SSc associated disorder. Anti-topisomerase, anti-Th/To, anti-PmScl, anti-U3RNP, anti-RNAPol I and anti-NOR90 can all be expected to produce nucleolar staining. More specifically, anti-Th/To, anti-PmScl produce homogenous nucleolar staining, anti-U3RNP clumpy nucleolar staining and anti-RNAPol I and anti-NOR90 punctate nucleolar staining. The pattern associated with anti-topoisomerase is considered distinctive in its own right. Anti-RNAPol I nearly always occurs in conjunction with anti-RNAPol III and we would also recommend further testing for this autoantibody in the appropriate clinical context. In practice, this pattern should result in further autoantibody testing with a panel of relevant SSc autoantibodies.
Speckle	Speckle patterns can be described as coarse or fine. These patterns are arguably the least specific and are also seen, usually at low titre, in healthy individuals. Fine speckle nucelar staining can be seen in a number of different autoimmune connective tissue diseases including SS, SLE, SSc and IIM. Further testing is likely to depend on the clinical context. Consistent with autoantibodies directed against Ro/La, Mi-2, TIF1γ, and Ku. Coarse speckled staining can be seen in a range of different autoimmune connective tissue diseases. Further testing is likely to depend on the clinical context. Consistent with autoantibodies directed against U1RNP, Sm, RNAPol III and Ku.
Cytoplasmic	
	Many IIM autoantibodies produce cytoplasmic standing patterns including, anti-synthetase autoantibodies, anti-SRP and anti-HMGCR. Not all laboratories will report these patterns as ANA positive. If IIM is a possible diagnosis we would recommend informing the laboratory and asking specifically about cytoplasmic staining. While distinct cytoplasmic patterns are described in practice we would recommend testing for all relevant myositis autoantibodies listed above.

Figure 1. Autoantibodies and their associated connective tissue diseases

Autoantibodies are considered highly specific for the associated connective tissue diseases. Where shown separately below they are typically mutually exclusive and where overlapping they may occur in combination. Autoantibodies which are particularly common within a connective tissue disease subgroup have been highlighted. Anti-Ro52 has been described in many of the connective tissue diseases and occurs in conjunction with other autoantibodies. The identification of anti-Ro52 should not preclude more extensive autoantibody screening.



IBM; Inclusion body myositis, SLE; systemic lupus erythematosus, SS; Sjögren's syndrome, MCTD; mixed connective tissue disease, SSc; systemic sclerosis,

SRP; anti-signal recognition peptide, HMGCR; anti-3-hydroxy-3-methyl-glutaryl-coenzyme A reductase, NXP2; anti- nuclear matrix protein 2, SAE; anti- small ubiquitin like modifier activating enzyme, Mi2; anti-Mi2, TIF1; anti- transcriptional intermediary factor 1 gamma, MDA5; anti- melanoma differentiation-associated protein 5, CN1A; anti- cytosolic 5′-nucleotidase 1a, Rib P; anti- ribosomal P proteins, DNA; anti double stranded DNA, La; anti-La/SSB, Ro; anti-Ro/SSA, U1RNP; anti-U1 ribonucleoprotein, Sm; anti-Smith, PmScl; Anti-polymyositis-scleroderma, Ku; anti-Ku, NOR90; anti- Nucleolar Organising Region 90, Jo-1; anti-Jo1/histidyl tRNA synthetase, OJ; anti-OJ/ isoleucyl-tRNA synthetase, Zo; anti-Zo/phenylalanyl tRNA synthetase, PL7; anti-PL7/threonyl-tRNA synthetase, KS; anti-KS/ asparaginyl-tRNA synthetase, EJ; anti-EJ/ glycyl-tRNA synthetase, Ha; anti-Ha/ Tyrosyl tRNA synthetase, PL12; anti-PL12/ Alanyl tRNA synthetase, U3RNP; anti-U3 ribonucleoprotein/fibrallin, EIF2B; anti- eukaryotic initiation factor 2B, RNAPol III; anti-RNA polymerase III, Topo I; anti-Topooisomerase I, Th/To; anti-Th/To, U11/U12 RNP; anti-U11 and U12 ribonucleoproteins, ACA; anti-centromere, aCL; anti-cardiolipin, LAC; lupus anti-coagulant, aβ2GPI; anti- β2glycoprotein I

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