The role of the clinical immunology laboratory in the diagnosis and monitoring of connective tissue diseases

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ABSTRACT

Connective tissue diseases (CTD) are a group of autoimmune systemic diseases that can affect any organ-system in the body. The initial clinical presentations of these diseases overlap, not only with each other, but also with a wide range of other rheumatological and non-rheumatological disorders. Due to these reasons, clinicians depend heavily on the use of the clinical immunology laboratory for the diagnosis of CTD. A large number of tests exist in the laboratory for the investigation of CTD and each test can be performed by a number of different methods, each with its own limitations. Consequently, the significance of the results generated not only has to be interpreted in relation to the clinical picture, but also to the method used to generate the results. Moreover, within the laboratory, there is a hierarchical testing system for the investigation of CTD and if this system is used appropriately, in conjunction with the clinical picture, can result in the diagnosis/exclusion of CTD more efficiently and economically. In contrast, random use of the laboratory tests, combined with limited knowledge of the methods used to carry out these tests, can lead to delay or even misdiagnosis, as well as can lead to wastage of resources. In the following review, we have discussed the various tests that are used in the investigation of CTD, as well as the different methods used to carry out these tests, with the hope that such knowledge would lead to a more efficient and economical use of the clinical immunology laboratory in the investigation of CTD.

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Connective tissue diseases (CTD) are a group of systemic autoimmune inflammatory diseases comprising of systemic lupus erythematosus (SLE), Sjogren's syndrome (SS), systemic sclerosis (Scl), Calcinosis, Raynaud's, Esophageal Dysmotility, Sclerodactyly and Telangiectasis (CREST) syndrome, polymyositis/dermatomyositis (PM/DM) and mixed connective tissue disease (MCTD).[1,2] Patients with CTD can present with clinical manifestations related to any organ-system of the body, often without the signs and symptoms that are classically associated with these diseases. However, the most common clinical presentation of CTD is with a rheumatological picture, often indistinguishable from that manifested by other more common rheumatological (such as osteoarthritis, rheumatoid arthritis, reactive arthritis) and non-rheumatological conditions (such as inflammatory bowel diseases, autoimmune hepatobiliary diseases, blood diseases, endocrine and metabolic diseases).[1-3]

In addition to being systemic diseases, CTD are associated with a wide range of autoantibodies that are directed against various components of the cell nucleus and are known as antinuclear antibodies (ANA).[4] The role of these antibodies in the pathogenesis of CTD is still controversial. However, their role in the diagnosis and prognosis of CTD is well established.[4-6] Antinuclear antibodies can present early in the course of the disease and

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therefore detection of these autoantibodies can be used in the early detection of CTD.[6,7] A wide range of tests have been developed for the detection of these autoantibodies, and are utilized extensively in the diagnosis and monitoring of CTD. Some of these tests can be used to screen patients presenting with common rheumatological manifestations for the possibility of CTD, while other tests are used to subdivide patients suspected of having these diseases.

Each test can be performed by a number of methods, each having varying degrees of sensitivities and specificities.[8,9] Consequently, the significance of the generated results depends, not only on the clinical picture, but also on the methods used to generate the results and positive and negative results do not always exclude or include the presence of CTD. For these reasons, good knowledge of the methods used in the clinical immunology laboratory for the investigation of CTD is essential for the proper interpretation of the generated results and thus for the diagnosis or exclusion of these diseases.

In the present review, we will discuss the different methods used in the clinical immunology laboratory for the investigation of CTD and comments on the results generated by each method with regards to the diagnosis or exclusion of CTD. We hope that this review would give clinicians a good insight into the various tests and methods that are available in the clinical immunology laboratory and help them focus their test-request and make proper interpretations of the generated results and thus lead to more efficient ways of diagnosing or exclusion of CTD. We also hope that the review would stimulate close clinical liaisons between clinicians and immunologists and lead to a more efficient and economical use of the clinical immunology laboratory in the investigation of CTD.

Antinuclear antibodies. Antinuclear antibodies are autoantibodies produced against various components of the cell nucleus. These antibodies include those directed against the double stranded- (or native)-DNA (dsDNA), the centromere and the extractable nuclear antigens (ENA).[5,6,10] Extractable nuclear antigens refer to a group of ribonuclear proteins (RNP) that can be extracted from the cell nucleus with saline solution. Six anti-ENA antibodies are commonly tested for and those include Ro (SS-A), La (SS-B), Smith antigen (Sm), scleroderma-70 (Scl-70), RNP and topoisomerase-1 (Jo-1). Although Jo-1 is largely found in the cytoplasm, it is still included amongst the ENA, since some of it also exists in the nucleus and can be extracted along with the rest of the antigens. Some of the ENA antibodies bear the initials of the names of patients (Ro, La, Sm, Jo-1) or the diseases (Scl-70; scleroderma, SS-A and SS-B; Sjogren's syndrome A and B) in which they were first described. Others have the name of the actual antigen (RNP).[4,5,11] Antinuclear antibodies are detected by an indirect immunofluorescence (IIF) method using animal tissues or human derived cell lines as substrates.[5] Binding of ANA to the various parts of the nucleus of the cell gives rise to a number of different immunofluorescence (IF) patterns. Some of these patterns are diagnostic of CTD,[10-12] while other patterns suggest the presence of specific ANA, which can be investigated further by other tests and used in the diagnosis of CTD (Ro, La, dsDNA).[5,13] However, in many instances, the IIF-patterns are not associated with any of the known ANA and their clinical significance remains unknown. In general however, presence of ANA-patterns strongly suggests the presence of CTD and further investigation should always be carried for more specific ANA.

Screening for antinuclear antibodies. Since CTD can present with the same manifestations as other common inflammatory and non-inflammatory rheumatological conditions, the use of simple, cheap, but sensitive screening methods is essential. Screening for ANA is performed by an IIF method using either rodent derived tissues (more commonly rat liver tissues) or human derived cells (Hep-2) as substrates.[14] Theses substrates have both advantages and disadvantages and the significance of the results generated will depend on the type of substrate used.[15,16]

Some of the ANA-patterns are diagnostic on their own rights (such as the nucleolar and the centromere patterns are diagnostic of systemic Sclerosis and CREST syndrome).[11,12,15] However, in the majority of cases, the patterns can direct further testing for more specific antibodies (**Table 1**). Rat liver tissues, which can be produced in house or bought commercially, are cheap, but less sensitive than Hep-2 cells. Some of the patterns, such as the centromere which is only present in dividing cells, cannot be detected in the rat liver tissues. Moreover, some of the antigens, particularly Ro, are either found in low titers or absent in these tissues.[5] Furthermore, both Ro and La antigens can leach out from the rat liver tissues during processing and thus can give rise to false negative results.[14,16,17] In our previous study, we showed that rat liver tissues could potentially miss out 25% of patient serum with antibodies to Ro antigen.[16] In contrast, Hep-2 cells are more sensitive and, with their large and dividing nuclei, can be used to detect the majority of antinuclear antibodies. The sensitivity of Hep-2 cells is further determined by the stage of cell growth at which they are prepared and the method used to fix them. Preparation of Hep-2 cells at the stationary phase would lead to the loss of the centromere pattern, while fixation can results in the alteration of the structures of some of the antigens, particularly the Ro antigen, and consequently failure

Table 1 - Antinuclear antibodies (ANA)-patterns and their association with different nuclear antigens and associated connective tissue diseases (CTD).

ANA-patterns	Corresponding antigens	Associated CTD
Homogeneous	dsDNA, Histones	SLE/drug induced SLE
Peripheral	dsDNA	SLE
Speckled	Sm RNP Ro/La Scl-70	SLE SLE/MCTD SS/SLE Scl
Discrete speckles	Centromere	CREST
Nuclear Membrane		PBC
Nuclear Pores		PBC
Nucleolar	Nucleolar RNA PM-Scl	Scl polymyositis-Scl
Nuclear dots	Sp100 (Nsp1), coilin p80	SS, SLE, PBC, AILD

dsDNA - double stranded-DNA, Sm - smith antigen, RNP - ribonuclear protein, Scl-70 - scleroderma-70, PM-Scl - polymyositis-scleroderma, SLE - systemic lupus erythematosous, SS - Sjogren's syndrome, RNA - ribonucleic acid Scl - systemic sclerosis, CREST - Clacinosis, Raynaud's, Oesophageal Dysmotility, Sclerodactyly and Telangiectasis, PBC - primary biliary cirrhosis, AILD - autoimmune liver diseases.

to detect them.[18] It is therefore important that laboratories test a number of different commercial preparations of Hep-2 cells before committing themselves to a particular preparation.

Antinuclear antibodies are found in varying percentages in different CTD, approaching 100% in SLE when Hep-2 cell line is used for screening (**Table 2**).[5,7] Antinuclear antibodies on their own are not diagnostic of CTD, as they can be found in other rheumatic and non-rheumatic conditions as well as in some healthy elderly people, albeit low titers.[19] However, high ANA titers are generally strong predictor for CTD and their presence should always lead to further investigation for more specific antibodies including those directed against ENA and dsDNA.

Certain nuclear patterns (such as nuclear membrane, pores and dots) may indicate the presence of other conditions beside CTD such primary biliary cirrhosis and autoimmune hepatitis.[5] Presence of such patterns should lead to testing for autoimmune hepatobiliary serology (smooth muscle and mitochondrial antibodies). Presence of strong unidentified ANA should be monitored frequently (such as 6-12 months) in order to check for the persistence or disappearance of these antibodies. Persistence of strong ANA suggests the presence of CTD and such patients should be monitored annually or as clinically indicated (along with other more specific ANA tests) for the possibility of future development of CTD. With regard to

Table 2 - Prevalence of ANA, dsDNA, ENA and histone antibodies in different CTD.

Disease	ANA	dsDNA	Ro	La	RNP	Sm	Scl-70	Jo-1	Hitones
SLE	95-100	60-80	35	15	30-40	30-40			18-50
Drug-induced SLE	100								95
SS	89-90		60	65					
Scl	97						30-40		
CREST	89								
PM/DM	40-80							20-40	
MCTD	95				90				
RA	30-50				50*				

ANA - antinuclear antibodies, dsDNA - double stranded-DNA, Sm - Smith antigen, ENA - extractable nuclear antigens, CTD - connective tissue diseases, RNP - ribonuclear protein, Scl-70 - scleroderma-70, SLE - systemic lupus erythematosus, SS - Sjogren's syndrome, Scl - systemic sclerosis, CREST - Clacinosis, Raynaud's, Oesophageal Dysmotility, Sclerodactyly and Telangiectasis, MCTD - mixed connective tissue disease, PM/DM - poly myositis/dermatomyositis, RA - rheumatoid arthritis. *not widely reported.

reporting of ANA results, since some of the patterns are diagnostic, ANA-titers as well as patterns should always be reported together.

Testing for antibodies to the extractable nuclear antigens. Extractable nuclear antigens (ENA) refer to nucleoproteins that can be extracted from the cell nucleus with saline.[5,9] They include Ro (SS-A), La (SS-B), RNP, Sm and Scl-70.[7,9,11,20] Jo-1, a histidyl-tRNA transferase, although a cytoplasmic antigen, is still included in the ENA family since small amount of it exists in the nucleus, which can be extracted along with other ENA.[21] Testing for antibodies to ENA is performed by a variety of different methods including the classic immunodiffusion and the counter current immuno-electrophoresis (CCIE) as well by enzyme-linked immunosorbent assay (ELISA) and Western blotting assays.[9,22,23] These different methods have different sensitivities and specificities for the detection of the different ENA antibodies and therefore for the diagnosis of CTD.[24,25]

Immunodiffusion and the CCIE are both cheap and highly specific for the detection of ENA antibodies. However, these methods are less sensitive and time consuming. Western blotting is both specific and sensitive, but is expensive, time consuming and requires good skill.[9] Enzyme-linked immunosorbent assays are highly sensitive, require very few skills and have a short turn around time. However, they are expensive and have been reported to be less specific than the above gel-assays.[26-28] For more detailed comparison of the different techniques used in the detection of ENA antibodies, the reader is referred to the review by Phan et al.[22]

In practice, only the CCIE and ELISA assays are routinely used for the detection and measurement of ENA antibodies.[20] The CCIE is highly specific for antibodies to ENA and positive results obtained by this method invariably indicate the presence of CTD.[9,25] However, because the method is time consuming and less sensitive, particularly for the detection of the Ro- and Scl-70 antibodies, many clinical immunology laboratories have switched to ELISA-assays.[23] Laboratories which continue to use the CCIE method have introduced, in addition, ELISA-assays for the detection of Scl-70. A number of strategies, involving combination of 2 or more methods, have been suggested for the detection of ENA antibodies.[23,29] One strategy involves screening for ENA antibodies by the CCIE and identifying specific antibodies by ELISA-assay, while another involves screening by ELISA-assays and identifying individual ENA antibodies by the CCIE-method.[23] Such strategies are based on the recommendation of the European consensus workshop which recommended the use of at least 2 methods for the detection of ENA antibodies.[29] In practice however, most of the clinical immunology laboratories use ELISA-assays for both screening and sub-typing of ENA antibodies.[23] Enzyme-linked immunosorbent assay-assays are reported to be less specific than the CCIE-method and this has led some clinicians to question the previously held view of the strong correlation between ENA-results and the presence of CTD. It is plausible however, that the specificity of ELISA-assays for ENA antibodies is dependent on the purity and the native structures of the ENA that are used to coat ELISA-plates.[9,22] Indeed, this would seem to be the case based on our previous small study where we showed that some ELISA-assays were less specific, while others were as specific as the CCIE-method.[16] Therefore, as with Hep-2 cell preparations, it is vital that laboratories considering introducing ELISA-assays, in place of the CCIE, test a number of different ELISA-products before committing themselves to a particular preparation. Enzyme-linked immunosorbent assay-assays can be used for both screening and identification of ENA antibodies. The CCIE method can be used when the results generated by the ELISA-assays are either weak positive or do not correlate with the clinical picture.

Regarding the interpretations of ENA-results, since a number of different methods are used for the measurement of ENA antibodies and since each assay has its own limitations, results generated must always be interpreted, not only in relation to the clinical picture, but also to the method used to generate such results and this would require close liaisons between requesting clinicians and the immunopathologist. Anti-extractable nuclear antigens-antibodies are used for the diagnosis and prognosis of CTD. The association between different ENA antibodies and different types of CTD is shown in **Table 2**.[4,6,13,30] Such association was originally deduced using the highly specific gel-assays (Immuno-diffusion and the CCIE-methods).[9] The individual ENA antibodies and their association with different CTD are discussed below.

Antibodies to Ro and La antigens. Anti-Ro and La antibodies were originally identified in patients with Sjogren's syndrome (SS) and are also called SS-A and SS-B.[11] These antibodies are associated with SS (occurring in both primary and secondary conditions) and to some extent SLE.[13,31] Anti-Ro and La antibodies have also been reported to occur in some patients with lymphoma.[32] In patients with SS, presence of anti-Ro and La antibodies are closely associated with development of extraglandular manifestations, (arthralgia, vasculitis, nephritis, lymphadenopathy and leucopenia) and such patients require close monitoring for the development of these associated conditions.[33,34] Unlike anti-Ro, anti-La antibodies have been reported to increase during flare up of SS and the concentrations correlate with lymphocyte

infiltration of the salivary glands.[34] In patients with SLE, presence of anti-Ro- and La antibodies are commonly associated with pneumonitis, photosensitive rash without severe renal disease, a condition known as subacute cutaneous SLE.[35]

Transfer of anti-Ro- and La antibodies across the placenta can lead to transient photosensitive rash and congenital heart block in the fetus.[36,37] Therefore, it is vital that pregnant women with SLE and SS be monitored for the presence of these antibodies and if present, performing fetal 4 chamber echocardiograms for the early detection of complete heart block and endocarditis.[30]

Antibodies to RNP antigen. The most important RNP antibodies are those directed against the U1-RNP.[30] These antibodies are associated with MCTD and SLE.[13,38] In the absence of dsDNA antibodies, anti-RNP antibodies are highly specific for MCTD.[39] Ribonuclear proteins antibodies have also been reported to occur in as many as 50% of patients with RA.[30] In a case report, RNP antibodies were also reported in a patient with undifferentiated carcinoma without any evidence of CTD.[40] However, such as data are not widely reported in the literature. It remains possible that these patients with RNP antibodies may go on to develop either MCTD or SLE. The level of anti-RNP antibodies in patients with MCTD or SLE does not change with the course of the disease, except when patients are on cytotoxic therapy (such as Cyclophosphamide, Azathioprine) when the titers may decrease or disappear altogether.[30] RNP-results produced by the CCIE-method are highly specific for SLE and MCTD. Results produced by ELISA-assays, particularly those with low levels, should be interpreted in relation to the clinical picture, since some of the ELISA-products can occasionally produce false-positive results.[16]

Antibodies to Sm antigen. Anti-Sm antibodies produced by the CCIE-method are highly specific for SLE and occur largely in patients of Afro-Caribbean origin.[41,42] These antibodies were given the first 2 letters of the first patient in whom they were first detected (Smith). Smith antigen antibodies are directed against a core protein of the nuclear RNP.[5] The antibodies tend to occur with RNP antibodies and it has been suggested that both antigens may share some antigenic epitopes.[41] Presence of Sm antibodies in patients with SLE is associated with renal disease and poor prognosis.[38,43] Unlike the CCIE method, ELISA-assays tend to produce false-positive results and this has led some clinicians to question the specificity of Sm antibodies for SLE.[9] It is therefore important that Sm results are interpreted in relation to the method used as well as the clinical picture.

Antibodies to Scl-70 antigen. Antibodies to Scl-70 are commonly detected by ELISA-assays, since the CCIE-method is less sensitive for the detection of these antibodies. Scleroderma-70 antigen has been shown to correspond to the DNA-topoisomersae-1, the enzyme responsible for decoiling of DNA during the process of transcription.[30,44] Anti-Scl-70 antibodies are associated with scleroderma. They occur in 20-40% of patients with systemic sclerosis and in 20 % of patients with limited scleroderma.[13,30] Patients with these antibodies tend to have poor prognosis of the disease as they develop cardiopulmonary disease and severe skin disease.[6,7] Anti-Scl-70 antibodies also occur in patients with the overlap syndrome, in which scleroderma is a part of the syndrome.[30]

In patients with Raynaud's phenomena, presence of anti-Scl-70 antibodies predicts a later development of progressive systemic Sclerosis.[7] The level of anti-Scl-70 antibodies does not seem to correlate with the disease activity.

Antibodies to PM/Scl (PM-1) antigen. Antibodies to PM/Scl antigen are detected by the CCIE method and are only measured in some immunology laboratories. These antibodies are found in patients with Myositis/Scleroderma overlap syndrome, but can occur to some extent in patients with Myositis or with Scleroderma. Presence of anti-PM/Scl antibodies is associated with increased risk of renal disease.[6,7,30]

Antibodies to Jo-1 antigen. A number of antibodies directed against a family of aminoacyl tRNA-synthetase are seen in patients with polymyositis/dermatomyositis.[21,45] However, only antibodies to the histidyl-tRNA synthetase (Jo-1) are measured routinely. Anti-Jo-1 antibodies are found in 15-30% of patients with autoimmune polymyositis and tend to predict an aggressive form of the disease, with arthralgia and interstitial lung involvement.[46] Therefore, patients with anti-Jo antibodies should have close monitoring of their pulmonary function for the early detection of lung involvement.[45] Of the antibody positive patients, 50% will present with myositis, 40% with dermatomyositis and 10% with myositis associated with other CTD.[30] Quantitative measurement of anti-Jo-1 antibodies may be of value, since the concentration has been reported to correlate with the disease activity.[30] Detection of anti-Jo-1 antibodies is performed by both CCIE- and ELISA-methods, although the former method is less sensitive.

Histone antibodies. Histone antibodies are detected by IIF-method using rat liver tissues or Hep-2 cell line as substrates, as well as by ELISA-assays. These antibodies are found in approximately 18-50% of patients with SLE and in more than 95% of patients with drug-induced SLE (such as procainamide, hydralazine).[7,47] In patients with drug-induced SLE, histone antibodies occur alone, whereas in patients with SLE, they occur with other autoantibodies including anti-dsDNA antibodies.[47,48] Patients with anti-histone antibodies

do not always develop SLE, since it has been reported that 50% of patients treated with procainamide develop anti-histone antibodies, but less than half of these patients actually develop clinical manifestations of SLE.[30] Diagnosis of drug-induced SLE is very important since, in the majority of patients, withdrawal of the causative drugs lead to complete resolution of the clinical manifestations of SLE within a few weeks.[30]

Antibodies to dsDNA. Antibodies to dsDNA are measured by a number of different methods including the radio-immuno assay (the far assay), IIF-method using Crithidia Lucillae as a substrate and by ELISA-assays. Anti-dsDNA antibodies exist in both low and high affinity forms.[49,50] High affinity antibodies are diagnostic of SLE and tend to predict a poor prognosis of the disease.[51] These antibodies are deposited in tissues, particularly the kidneys, and lead to complement fixation and consequently organ damage. In contrast, low affinity dsDNA antibodies occur with a variety of other conditions and are not diagnostic of SLE. Moreover, such antibodies are not associated with complement consumption or organ damage.[16,51] The far assay is both sensitive and specific for the high affinity dsDNA antibodies and positive results are diagnostic of SLE. The IIF-method using Crithidia Lucillae is also specific, but less sensitive, for the detection of high affinity dsDNA antibodies and positive results are also diagnostic of SLE.[52] In contrast, ELISA-assays tend to detect both high and low affinity anti-dsDNA antibodies and results generated are not specific for SLE, as they can occur with a variety of other conditions.[16,51] It is therefore important that the results generated by ELISA-assays are interpreted in relation to the clinical picture.

The strategy used to measure dsDNA antibodies varies between laboratories. Some laboratories screen for dsDNA antibodies by the IIF-Crithidia Lucillae method and positive samples are then quantified by ELISA-assays, whereas others screen by ELISA-assays and confirm by the IIF-Crithidia Lucillae method. However, since the IIF-Crithidia Lucillae method is insensitive for the detection of dsDNA-antibodies, screening by this method could lead to generation of false-negative results, while using ELISA-assays for screening would lead to generation of a high number of positive results that would need to be confirmed by the IIF-Crithidae Lucillae method and thus lead to wasteful of resources. It may be necessary to use a third type of strategy in which screening for dsDNA antibodies is carried out by ELISA-assays and positive results of certain values go on to be confirmed by the IIF-Crithidia Lucillae method. Such strategy would be supported by our previous study where we found that positive results produced by ELISA-assays with values of >100 IU/ml were generally associated with SLE, whereas results of less than 100 IU/ml were associated with a variety of other conditions.[16] Rising titers of high affinity anti-dsDNA antibodies tend to predict a flare up of SLE and increase the risk of renal impairment.[51] Therefore, close monitoring of patients with SLE for anti-dsDNA antibodies is very important and detection of rising titers should lead to assessment of complement components and renal function.

Rheumatoid factor. Rheumatoid factor (RF) is an auto-antibody with specificity for the constant-fragment (FC) of the human immunoglobulin (Ig)G-class. They can occur in all the different forms of the Ig-classes (IgG, IgM, IgA, IgE, IgD). However, only the IgM class is measured clinically.[30] Rheumatoid factor is classically associated with RA and is used as a criteria in the diagnosis of this disease.[53] However, since 10% of patients with RA lack RF and since it is found in a variety of different conditions, including CTD (**Table 3**), its presence or absence per se neither implicate nor exclude the diagnosis of RA.[7,54] However, many clinicians still regards the presence of RF as an indication for RA. In a recent survey, many general practitioners referred patients to the Rheumatologists based on the presence of positive RF-results.[55] Presence of RF in clinically diagnosed patients with RA serve as a prognostic indicator of the disease and high titers at presentation predict a severe form of RA, with extra-articular

Table 3 - Prevalence of RF in CTD and other diseases.

Disease	Prevalence
Rheumatoid arthritis	50-90
Sjogren's syndrome	70-90
Systemic lupus erythematosus	15-35
Systemic sclerosis	20-30
Juvenile rheumatoid arthritis	7-10
Polymyositis	5-10
Others	
Infections	*
Hepatitis C infection	
Infectious mononucleosis	
Acute viral infection	
Tuberculosis	
Leprosy	
Infective endocarditis	
Syphilis	
Chronic liver disease	*
Sarcoidosis	*
Cryoglobulinemia	*
Elderly people	1-5

*percentage of patients with unknown RF
RF - rheumatoid factor, CTD - connective tissue diseases

manifestations.[56,57] Such patients need prompt referral to Rheumatologists for close monitoring of the disease and institution of aggressive therapy.

Presence of RF in patients with CTD can be associated with type-II and type-III cryoglobulinemia. Rheumatoid factor tends to be requested, along with other autoimmune tests, for screening of CTD which may serve as a pointer towards RA in the absence of CTD positive serology, or as a possible indicator for the future development of cryoglobulinemia in patients with CTD. Traditionally, screening for RF has been carried out by the latex agglutination method. This method is simple, cheap and very sensitive for the detection of RF. Positive samples are then quantitated by the Rose Waalar method using sheep-RBC agglutination test. However, the latter method is labor intensive and involves a number of steps which can contribute to the imprecision of the end results. For these reasons, it has been replaced by ELISA-assays and by immuno-nephelometric/ turbidimetric tests that are performed by automated analyzers. The latter methods are highly sensitive and produce quantitative results in IU/ml. However, since RF test are among the most requested test in the immunology laboratory, the use of the latter methods can be expensive.

The choice of the assays used for measurements of RF would depend on the number of tests performed and on the budget of the laboratory. For laboratories with limited resources, it is possible to use a combination of the simple latex agglutination method and ELISA assays. Screening could be carried out using a simple inexpensive latex agglutination assay and positive samples are then quantitated by an ELISA-assay.

Anticardiolipin antibodies. Anticardiolipin antibodies (ACLA) are a group of heterogeneous antibodies directed against a number of phospholipids which include cardiolipins.[48] Anticardiolipin antibodies, also termed antiphospholipid antibodies, are associated with the antiphospholipid syndrome (APS).[58] The syndrome is characterized by recurrent venous and arterial thrombosis, recurrent fetal loss, stroke, livedo reticularis and thrombocytopenia.[59] Antiphospholipid syndrome can occur either as a primary syndrome or as a secondary syndrome in patients with CTD. Anticardiolipin antibodies are also found in conditions other than APS, including infections (hepatitis C, leprosy, Lyme disease, Q fever, varicella zoster, tuberculosis, human immunodeficiency virus and legionaries disease), leukemia and solid malignancies. However, in these latter conditions, the antibodies are not associated with the clinical manifestations of the APS.[60] Anticardiolipin antibodies are measured by ELISA-assays and by a functional clotting assay termed lupus anticoagulant. Recently, ACLA associated with the APS were reported to recognize mainly the cardiolipin binding protein [beta-2-glycoprotein 1(ß2GP1)]. The antibodies were reported to recognize epitopes on the ß2GP1, epitopes shared by the cardiolipins and the ß2GP1 molecules as well as conformational epitopes located on the ß2GP1 molecules. The latter epitopes are induced as a result of binding of ß2GPIb to cardiolipin molecules.[61] For this reason, recently ß2GP1b has been added to ELISA-plates either as a secondary coating or with the diluents/washing solutions. While ACLA associated with APS are directed against the ß2GP1b molecules, those found in infections and other conditions are directed against the phospholipids molecules.[60] Anticardiolipin antibodies are found in 20-40% of patients with SLE who are at risk of developing secondary APS.[58,59] Since ACLA that are associated with infections and with other CTD are not clinically significant, it may be advisable not to include the ACL-test amongst the initial tests used to screen patients for CTD, unless clinically indicated. However, the ACL-test should be performed in all patients with positive SLE serology so that patients with these antibodies may be closely monitored for the future development of clinical manifestations of the APS.

Anticardiolipin antibodies are also detected by another test termed Lupus-anticoagulant test (LAT), manifested in-vitro by prolonged activated partial thromboplastin time and corrected with addition of phospholipids.[62] Since, ACLA detected by both assays are directed against different parts of the cardiolipin/ß2GP1 molecules, and do not necessary coexist, both assays should be used simultaneously for the detection of these antibodies.

Enzyme-linked immunosorbent assays measure both IgG and IgM classes of ACLA. The IgG class is more significant than the IgM, while the significance of low IgM ACLA is unclear. However, in rare cases, the symptoms of the APS can be associated with IgM antibodies alone.[63] Results produced by the Lupus anticoagulant test are reported to be more significant than the ELISA-results.[64]

Initial positive results by both ELISA and the Lupus anticoagulant tests should be repeated (such as 6-8 months) to check for persistence of the ACLA, since persistent high titers suggest increased risk of developing APS. High titers of ACLA do not seem to correlate with the severity of the symptom.[30] Patients with SLE who are planning for pregnancy should be screened for both anti-ACLA as well as for anti-Ro and La antibodies.

Anti-neutrophil cytoplasmic antibodies. Anti-neutrophil cytoplasmic antibodies (ANCA) are antibodies directed against a range of neutrophil cytoplasmic components (enzymes and proteins).[65] Testing for ANCA is indicated in patients suspected

of having vasculitis.[66] Screening for ANCA is performed by IIF-method using ethanol fixed human neutrophils as substrate. Two IIF-patterns are observed, a peri-nuclear (P-ANCA) and a cytoplasmic (C-ANCA) patterns. P-ANCA is due to antibodies binding to a number of negatively charged cytoplasmic enzymes and proteins (including Myeloperoxidase [MPO], lysozyme and lactoferrin) that are induced to accumulate around the positively charged nuclear membrane by ethanol during the fixation stage. In contrast, the C-ANCA pattern is due to antibodies binding to proteinase-3 (PR3) enzyme.[67] Positive ANCA-results should always lead to further investigation and quantifications of these cytoplasmic enzymes by ELISA-assays. For practical purposes, many laboratories only measure MPO and PR3 enzymes.[65] Cytoplasmic-ANCA and PR3 results are strongly associated with Wegener's granulomatosis (WG) occurring in 60-90% of patients. In addition, they are found in 25-30% of patients with microscopic polyangiitis, 30% in patients with Churg Strauss syndrome and in patients with rapidly progressive glomerulonephritis.[65,67] However, the latter condition has been suggested by some authors to be a limited form of WG, rather than a separate disease entity.[65] Positive P-ANCA and MPO results are associated with microscopic polyangiitis, Churg Strauss syndrome, pauci-immune crescentic glomerulonephritis and anti-glomerular basement disease. Positive P-ANCA-MPO results are also found in a small number of patients with RA and SLE.[68] Positive C-ANCA or P-ANCA, in the absence of positive enzymes, are non-specific results and occur with a wide variety of conditions including CTD.[65,69]

Quantification by ELISA-assay is used for monitoring of patients with WG, as falling PR3 levels is associated with good response to treatment while rising levels, in patients on remission, predict a flare up of the disease.[70] Although positive ANCA-results can be associated with CTD, they do not add any useful clinical information to the diagnosis or prognosis of these diseases. However, since presentation of CTD and systemic vasculitis can overlap on occasions, ANCA tests are added to the list of CTD tests in order to exclude the possibility of ANCA-positive vasculitis. Moreover, in patients with CTD who go on to develop vasculitis or glomerulonephritis, it would be appropriate to request ANCA-tests in order to exclude the possibility of ANCA-positive vasculitis.

Other immunological tests used in the investigation of CTD. ***a) Immunoglobulin levels.*** Quantitative as well as qualitative measurements of serum Igs (IgG, IgA, IgM) are essential part of the investigation for CTD. Quantitative measurement is performed more commonly by nephelometric/turbidimetric assays using automated analyzers, but can be measured by other assays including ELISA and radial immuno-diffusion assays. Reduction of Igs can occur with malignancies (Myeloma/Lymphoproliferative disease) which can present with musculoskeletal symptoms similar to CTD and other rheumatological conditions. Reduction of Igs can also occur with protein loss (gut/kidneys) and primary immuno-deficiencies.[7] Increased level of serum polyclonal Igs is a feature of CTD, but can also occur with a variety of other conditions.[71] In CTD, particularly SS, increased polyclonal Igs is due to the massive production of the IgG1 subclass.[72] Qualitative measurement is more commonly performed by the capillary zone electrophoresis and paraproteins identified by immuno-fixation. Qualitative measurements of Igs reveal presence of paraproteins in serum. Presence of paraproteins in patients with CTD is common and they do not necessarily indicate the presence of malignant conditions (Myeloma/LPD).[73] However, since paraproteins are commonly associated with malignancies and which, on occasions, can present with a musculoskeletal picture, detection of paraproteins in patients suspected of or having CTD should lead to exclusion of malignant conditions. Paraproteins associated with CTD are benign form of paraproteinemia. However, since there is always a risk of developing to frank lymphoma, these paraproteins should be monitored indefinitely. This risk of transformation is particularly high in patients with Sjogren's syndrome, and some authors have referred to this disease as a pre-malignant condition.[74] Monitoring of Igs, both quantitatively and qualitatively, is performed routinely in patients with CTD who are on immunosuppressive therapy for the early detection of secondary immunodeficiency.[75,76]

b) Complement levels. Testing for complement levels, particularly C3 and C4, are included among the tests performed for CTD. Low C3 and C4 are found in patients with SLE, and rarely in patients with RA with vasculitis.[77] High affinity anti-dsDNA antibodies (markers for SLE) are deposited in tissues, particularly the glomeruli, and cause complement fixation with consequent decreases in C3 and C4 levels and increased risk of renal impairment.[78] Deficiency of C4 is more common in patients with SLE and low C4 alone does not necessarily indicate complement consumption.[80] Reduction of complements also occurs in the presence of cryoglobulins which can develop in patients with CTD (secondary cryoglobulinemia).[81] Therefore, low complement levels with or without low IgG levels should lead to testing for cryoglobulins. Other conditions associated with complement reduction are listed in **Table 4.** Complement components are measured as for Igs.

Table 4 - Conditions associated with reduction in complement levels.

Low C4, Low C3	Low C4, Normal C3	Normal C4, Low C3
Active SLE	Hereditary C4 deficiency	Post-streptococcal GN
RA with vasculitis	Hereditary angioedema	C3 nephritic factor
Sepsis	Active SLE	Gram negative sepsis
Severe liver disease	Type II cryoglobulinemia	MPGN
Protein loss (gut/kidneys)	Acquired angioedema	SBE
Cryoglobulinemic nephritis	(SLE, Lymphoma)	
Shunt nephritis	Eclampsia	
Nephritis of SBE		
Penicillamine nephritis		
Severe hemolysis (warm antibody)		

C - complement, SLE - systemic lupus erythematosus,
RA - rheumatoid arthritis,
GN - glomerulonephritis,
MPGN - membranoproliferative glomerulonephritis,
SBE - sub-acute bacterial endocarditis.

Table 5 - Types of cryoglobulinemia and their associated conditions.

Types of cryoglobulinemia	Associated conditions
Type-1 monoclonal protein, usually IgM	LPD, WMG Myeloma,
Type-2 monoclonal protein, usually IgM with RF activity and polyclonal IgG	LPD, WMG, Myeloma chronic infections (HCV,SBE), CTD
Type-3 polyclonal IgM with RF activity and polyclonal IgG	chronic infections, RA CTD (SLE, SS), sarcoid

Ig - immunoglobulin, LPD - lymphoproliferative disease,
WMG - Waldenstroms's Macroglobulinemia,
HCV - Hepatitis C virus,
SBE - sub-acute bacterial endocarditis,
CTD - connective tissue disease,
SLE - systemic lupus, erythematosus, RF - rheumatoid factor
SS - Sjogren's syndrome, RA - rheumatoid arthritis.

c) Cryoglobulins. Cryoglobulins are Igs that precipitate at low temperatures and re-dissolve at high temperatures. For the detection of cryoglobulins, blood is taken at 37˚C and maintained at this temperature until the blood has clotted and serum separated. Serum is then incubated at 4˚C for 48 hours and if cryoglobulins are present, precipitates are formed which tend to re-dissolve on re-warming the serum back to 37˚C.[7] Failure to maintain the blood at 37˚C can lead to precipitation of cryoglobulins and their subsequent loss during blood separation, giving rise to false-negative results. There are 3 types of cryoglobulins. Type 1 cryoglobulins are due to paraproteins, usually of the IgM isotype, and are associated with malignancies (myeloma/lymphoma, wadestrom's macroglobulinemia). Type 2 cryoglobulins consist of a paraprotein, usually IgM, with RF activity against polyclonal IgG. This type of cryoglobulins tend to associate with hepatitis C infection, lymphoma, CLL, Myeloma, WMG and CTD. Type 3 cryoglobulins is due to polyclonal IgM, with RF activity, and polyclonal IgG and tends to associate with hepatitis C infection and CTD (RA and SLE) (**Table 5**).[7,80,81]

Clinical presentation of cryoglobulinemia is quite wide and includes vasculitis, infarcts, renal involvement and musculoskeletal picture and thus can mimic CTD.[80] In patients with CTD, intolerance to cold with pain in exposed areas, presence of Raynaud's phenomenon, cutaneous vasculitis (purpura, urticaria, ulcers), glomerulonephritis and reduction of complements with or without the concomitant reduction of Igs, should lead to testing for cryoglobulins.[82]

Pattern of test-requests. The clinical immunology laboratory provides a wide range of tests for the investigation of CTD. Some of these tests are used to screen for the presence of CTD, while others are used in the diagnosis of the individual diseases within the CTD group. Connective tissue disease can present with clinical manifestations similar to that manifested by some of the more common rheumatological diseases. Therefore, it would be necessary to undertake some form of investigations in patients presenting with rheumatological manifestations in order to rule out the presence of CTD. However, carrying out full investigation for CTD in all patients presenting to the general practitioners with rheumatological manifestations would be unnecessary and economically unviable. In contrast, failing to carry out any investigations for CTD in patients in whom the probability of CTD is low could potentially lead to delay in the diagnosis of CTD. In this section, we have outlined a possible chart to follow when investigating for the possibility of CTD. We hope that following this chart would allow screening patients for CTD to be carried out at an

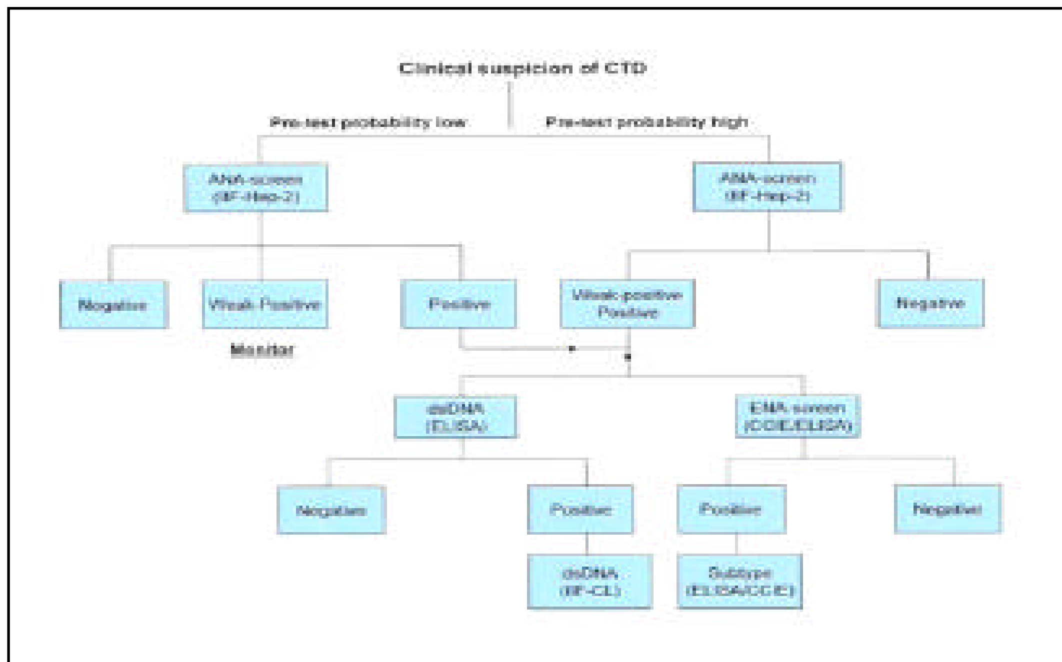

Figure 1 - Suggested flowchart for the laboratory investigation of connective tissue diseases (CTD). ANA - anti-nuclear antibodies, ENA - extractable nuclear antigens, IIF - Indirect Immunoflourescnece, CCIE - counter current Immuno-electrophoresis, CL - Crithidiae Lucillae, dsDNA - double stranded-deoxyribo-nucleic acid, ELISA - enzyme-linked immunosorbent assay, Hep-2 - human derived cells

economically acceptable cost (**Figure 1**). Although, the number of tests requested and the timing of such requests would be largely dictated by the clinical situation, this guide may help to out line how test-requests may be utilized optimally when investigating for CTD. Samples originating from the general practitioners, where the pre-test probability for CTD is low, could be screened for RF and for ANA (provided Hep-2 cell line is used for ANA screening). Samples with positive ANA results should be further investigated for more specific antibodies including ENA- and dsDNA antibodies, while samples with weak positive ANA could be monitored. Screening for ENA antibodies using the CCIE method should, if clinically indicated, lead to simultaneous testing for the Scl-70 and Jo-1 antibodies by ELISA-assays. Positive screen for ENA antibodies should lead to typing of the different ENA antibodies (including those directed against Ro, La, Sm, Scl-70 and RNP) using either the CCIE or ELISA-assays and guided by the clinical picture. Screening for dsDNA antibodies could initially be performed by ELISA-assays and positive samples are then further tested for the high affinity dsDNA antibodies by the IIF-Crithidia Lucillae method. Test request by the specialists would vary from that of the general practitioners and would also be guided by the clinical picture. However, it may be necessary on the first occasion to request most of the above tests in order to confirm or rule out the possibility of CTD. It may also be necessary to repeat the same battery of tests regularly (6-12 months) in patients with undifferentiated CTD, in order to check for future differentiation of these diseases. For monitoring purposes, dsDNA antibodies and complement measurements would be essential. Monitoring of Ig in patients with immunosuppressive therapy would also be important for the early detection of secondary immunodeficiency.

Recent expansion in the number of tests and methods in the clinical immunology laboratory for the investigation of CTD has helped clinicians to exclude, or diagnose and monitor these disorders more readily. However, since the number of test available is large and since each tests can be carried out by a variety of different methods, each with its own limitations, good knowledge of these tests and methods is essential for the efficient and more economical use of the clinical immunology laboratory in the investigation of CTD. We hope that we have provided some of that needed knowledge in the present review.

References

1. Kumar P, Clark M. Clinical Medicine. 5th ed. Edinburgh: WB Saunders; 2002. p. 511-586.
2. Shmerling RH, Fuches HA, Lorish CD, Nichols LA, Partridge AJ, Brigham RB et al. Guidelines for the initial evaluation of the adult patients with acute musculoskeletal symptoms. ***Arthritis Rheum*** 1996; 39: 1-8.

3. Chapel H, Haeney M, Misbah S, Snowden N. Clinical Immunology. 4th ed. Blackwell Science 1999. p. 183-205.
4. Swaak AJ, Huysen V, Smeenk RJ. Antinuclear antibodies in routine analysis: the relevance of putative clinical association. ***Ann Rheum Dis*** 1993; 52: 110-114.
5. Hollingsworth PN, Pummer SC, Dawkins RL. Antinuclear antibodies. In: Peter PN, Shoenfield Y, editors. Autoantibodies. Elsevier Science B V; 1996. p. 74-90.
6. White RH, Robbins DL. Clinical significance and interpretation of antinuclear antibodies. ***West J Med*** 1987; 147: 210-213.
7. Osterland CK. Laboratory diagnosis and monitoring in chronic systemic autoimmune diseases. ***Clin Chem*** 1994; 40: 2146-2154.
8. Feltkamp TEW, Klein F, Janssens MB. Standardization of the quantitative determination of antinuclear antibodies (ANAs) with a homogenous pattern. ***Ann Rheum Dis*** 1988; 47: 906-909.
9. Tan TE. Antinuclear antibodies: diagnostic markers for autoimmune disease and probes for cell biology. ***Adv Immunol*** 1989; 44: 93-151.
10. Takahashi M, Okada J, Kondo H. Six cases positive for anti-centromere antibodies with ulcer and gangrene in extremities. ***Br J Rheumatol*** 1997; 36: 889-893.
11. Fritzler MJ, Kinsella TD. The CREST syndrome: a distinct serological entity with anticentromere antibodies. ***Am J Med*** 1980; 69: 520-526.
12. Reimer G, Steen VM, Penning CA. Correlates between autoantibodies to nucleolar antigen and clinical features in patients with systemic sclerosis (Scleroderma). ***Arthritis Rheum*** 1988; 31: 525-532.
13. Hamburger HA. Cascade testing for autoantibodies in connective tissue diseases. ***Mayo Clin Proc*** 1995; 70: 183-184.
14. Kozin F, Fowler M, Koethe SM. A comparison of the sensitivities and specificities of different substrate for the fluorescent anti-nuclear antibody test. ***Am J Clin Pathol*** 1980; 74: 785-790.
15. Kern P, Kron M, Hiesche K. Measurement of antinuclear antibodies: Assessment of different system. ***Clin Diag Lab Immunol*** 2000; 7: 72-78.
16. Aziz KA. Ineffectiveness of rat liver tissues in the diagnosis of connective tissue diseases. ***Saudi Med J*** 2004; 25: 442-446.
17. Hormon CE, Deng J, Peebles CL, Tan EM. The importance of tissue substrate in the SS-A/Ro antigen-antibody system. ***Arth Rheum*** 1984; 27: 166-173.
18. Du L, Fukushima S, Sallmyr A, Manthorpe R, Bredberg A. Exposure of Hep-2 cells to stress condition influences antinuclear antibody reactivity. ***Clin Diag Lab Immunol*** 2002; 9: 287-294.
19. Juby AG, Davies P, McElhaney JE, Gravenstein S. Prevalence of selected autoantibodies in different elderly subpopulations. ***Br J Rheum*** 1994; 33: 1121-1124.
20. Hamburger M, Hodes S, Barland P. The incidence and clinical significance of antibodies to the extractable nuclear antigens. ***Am J Med Sci*** 1977; 273: 21-28.
21. Nishikai M, Reechlin M Heterogeneity of precipitating antibodies in polymyositis and dermatomyositis: characterization of Jo-1 antibody system. ***Arth Rheum*** 1980; 23: 881-888.
22. Phan TG, Wong RCCW, Adelstein S. Autoantibodies to extractable nuclear antigens: making detection and interpretation more meaningful. ***Clin Diag Lab Immunol*** 2002; 9: 1-7.
23. Rondeel JM, Gelder WV, Leeden HVD. Different strategies in the laboratory diagnosis of autoimmune disease: immunofluorescence, enzyme-linked immunosorbent assay or both? ***Ann Clin Biochem*** 1999; 36:189-195.
24. Lock JR. Unsworth DJ. Antibodies to extractable nuclear antigens. Has technological drift affected clinical interpretation? ***J Clin Pathol*** 54: 187-190.
25. Maddison PJ, Skinner RP, Valachyiannapoulos P. Antibodies to nRNP, Sm, Ro (SSA) and La (SSB) detected by ELISA: their specificity and inter-relations in connective tissue disease sera. ***Clin Exp Immunol*** 1985; 62: 337-345.
26. Meilof JF, Bantjes I, De Jong J. The detection of anti-Ro/SSA and anti-La/SSB antibodies. Comparison of counter immunoelectrophoresis with immunoblot, ELISA, and RNA precipitation assays. ***J Immunol Methods*** 1990; 133: 215-226.
27. James K, Meek G. Evaluation of commercial enzyme immuno assays compared to immunofluorescence and double diffusion for antibodies associated with autoimmune disease. ***Am J Clin Path*** 1992; 97: 559-565.
28. Monce NM, Bogusky RT, Cappel NN. An enzyme immunoassay screening test for the detection of total antinuclear antibodies. ***J Clin Lab Ana*** 1991; 5: 439-442.
29. van Venrooij WJ, Charles P, Maini. The consensus workshops for the detection of autoantibodies to intracellular antigens in rheumatic disease. ***J Immunol Methods*** 1991; 140: 181-189.
30. Ward AM, Wild GD, Riches PG, Sheldon J. PRU handbook of autoimmunity, PRU publications 2001. p. 89-134.
31. Wharen M, Tengner P, Gunnarson I, Lundberg I, Hedfors E, Ringertz NR et al. Ro/SSA and La/SSB antibody level variation in patients with Sjogren's syndrome and systemic lupus erythematosus. ***J Immunol*** 1998; 11: 29-38.
32. Sissa M, Amital-Teplizki H, Haim N, Cohen Y, Shoenfeld Y. Autoantibodies in neoplasia. An unresolved enigma. ***Cancer*** 1990; 65: 2554-2558.
33. Davidson BKS, Kelly CA, Griffiths ID. Primary Sjogren's syndrome in the north of England: a long-term follow up. ***Rheum*** 1999; 38: 245-253.
34 James JA, Dicky DJD, Fujsaku A. Antigenicity of recombinant Ro (SS-A) fusion protein. ***Arth Rheum*** 1990; 33: 102-106.
35. Sontheimer RD, Madisson PJ, Reichlin M, Jorden RE, Stasthy P, Gilliam JN. Serological and HAL associations in subacute cutaneous lupus erythematosus. ***Ann Intern Med*** 1982; 97: 664-671.
36. Singsen BH, Akhter JE, Weinstein MM, Sharp GC. Congenital complete heart block and SSA antibodies: obstetric complications. ***Am J Obstet Gynecol*** 1985; 512: 665-669.
37. Marvragani CP, Dafni UG, Tzioufas AG, Moutsopoulos HM. Pregnancy outcome and anti-Ro/SSA in autoimmune disease: a retrospective cohort study. ***Br J Rheum*** 1998; 37: 740-745.
38. Holyst MM, Hoffman RW. U-small nuclear ribonucleoprotein (RNP) reactive autoantibodies: Diagnostic test and clinical interpretation. ***Clin Immunol Let*** 1998; 18: 53-57.
39. Panush RS, Edwards NG, Longley S, Webster E. Rhupus syndrome. ***Arch Intern Med*** 1988; 148: 1633-1636.
40. Foster HE, Malleston PN, Petty RE, Cabral DA. Anti-RNP antibody in a child with undifferentiated carcinoma and no evidence of mixed connective tissue disease. ***Br J Rheum*** 1997; 36: 289-291.
41. Arnett FC, Hamilton RG, Roebber MG. Increased frequency of Sm and nRNP autoantibodies in American blacks compared to whites with systemic lupus erythematosus. ***J Rheum*** 1988; 15: 1773-1776.
42. ter Borg EJ, Groen H, Horst G. Clinical association of anti-ribonucleoprotein antibodies in patients with systemic lupus erythematosus. ***Sem Arth Rheum*** 1990; 20: 164-173.
43. Worrall J, Snaith ML, Batchelor R, Isenberg DA. SLE-rheumatological view. ***Q J Med*** 1990; 275: 319-330.
44. Jacobsen S, Halgerg P, Ullman S, Van Verooj WJ, Hoier-Madsen M, Wjik A. Clinical features and serum anti-nuclear antibodies in 230 Danish patients with systemic sclerosis. ***Br J Rhuem*** 1998; 37: 39-45.

45. Bernstein RM, Morgan SH, Chapman J. Anti-Jo-1 antibody: a marker for myositis with interstitial lung disease. ***B M J*** 1984; 289: 151-152.
46. Marguerie C, Bunn CC, Byeynon HL, Bernstein RM, Highes JM, So AK. Polymositis, pulmonary fibrosis and autoantibodies to aminoacyl-tRNA synthetase enzyme. ***Q J Med*** 1990; 77: 1019-1038.
47. Rubin RL, Waga S. Antihistone antibodies in systemic lupus erythematosus. ***J Rheum*** 1987; 14 (Suppl 13): 118-126.
48. Spickett G. Oxford Handbook of Clinical Immunology. Oxford (UK): Oxford University Press; 1999.
49. Barbas SM, Ditzel HJ, Salonen EM, Wang WP, Sliverman GJ, Burton DR. Human antibody recognition of DNA. ***Proc Natl Acad Sci USA*** 1995; 92: 2529-2533.
50. Swaak T, Smeenk RJ. Detection of anti-dsDNA as a diagnostic tool: a prospective study in 441 non systemic lupus erythematosus patients with anti-dsDNA antibody (anti-dsDNA). ***Annal Rheum Dis*** 1985; 44: 245-251.
51. ter Borg EJ, Horst G, Hummell EJ. Measurement of increases in anti-double-stranded DNA antibody levels as a predictor of disease exacerbation in systemic lupus erythematosus. A long term prospective study. ***Arth Rheum*** 1990; 33: 634-643.
52. Davis P, Christian B, Russell AS. Immunofluorescence technique for the detection of antibodies to n-DNA: comparison with radioimmunoassay. ***J Rheum*** 1977; 4: 14-20.
53. Arnett FC, Edworthy SM, Bloch DA, Mcshane DJ, Fries JF, Cooper NS et al. The American rheumatism association 1987 revised criteria for the classification of rheumatoid arthritis. ***Arth Rheum*** 1988; 88: 315-324.
54. Saraux A, Guedes C, Allain A, Baron D, Youinou P, Le Goff P. From classification criteria to diagnostic criteria for rheumatoid arthritis. ***Rev Rheum*** 1996; 63: 159-161.
55. Sinclair D, Hull RG. Why do general practitioners request rheumatoid factor? A study of symptoms, requesting patterns and patients outcome. ***Ann Clin Biochem*** 2003; 40: 131-137.
56. Sherrer YS, Bloch DA, Mitchell DM, Young DY, Fries JF. The development of disability in rheumatoid arthritis. ***Arth Rheum*** 1986; 26: 494-500.
57. Feigenbaum SL, Masi AT, Kaplan SB. Prognosis in rheumatoid arthritis. A longitudinal study of newly diagnosed younger adult patients. ***Am J Med*** 1979; 66: 377-378.
58. Kandiah DD, Sali A, Sheng Y, Victoria EJ, Marquis DM, Coutts SM et al. Current insights into the "phospholipid" syndrome: clinical, immunological and molecular aspects. ***Adv Immunol*** 1998; 70: 507-563.
59. Hughes G. The anticardiolipin syndrome. ***Clin Exp Rheum*** 1985; 3: 285-286.
60. Hunt JE, McNeil HP, Morgan GJ, Crameri RM, Krillis SA. A phospholipid-ß2-glycoprotein 1 complex is antigen for anticardiolipin antibodies occurring in autoimmune disease but not with infection. ***Lupus*** 1992; 1: 75-81.
61. Hunt JE, Simpson RJ, Krillis SA. Identification of a region of ß2-glycoprotein 1 critical for lipid binding and anti-cardiolipin antibody cofactor. ***Pro Natl Acad Sci (USA)*** 1993; 90: 2141-2145.
62. Bevers EM, Galli M, Barbui T, Comfurius P, Zwaal RFA. Lupus anticoagulant IgG's (LA) are not directed to phospholipids only, but to a complex of lipid-bound human prothrombin. ***Throm Haemostasis*** 1991; 66: 629-632.
63. Reddel SW, Krills S. Testing for and clinical significance of anticardiolipin antibodies. ***Clin Diag Lab Immunol*** 1999; 6: 775-782.
64. Amengual O, Atsumi T, Khamashta MA, Koihe T, Hughes GRV. Specificity of ELISA for antibody to ß2-glycoprotein 1 in patients with antiphospholipid syndrome. ***Br J Rheum*** 1996; 35: 1239-1243.
65. Hoffman GS, Specks U. Antineutrophil cytoplasmic antibodies. ***Arth Rheum*** 1998; 41: 1521-1537.
66. Vassilpoulos D, Hoffman GS. Clinical utility of testing for antineutrophil cytoplasmic antibodies. ***Clin Diag Lab Immunol*** 1999; 6: 645-651.
67. Koldingsnes W, Gran JT, Omdal R, Husby G. Wegeners granulomatosis: long-term follow-up of patients treated with pulse cyclophosphamide. ***Br J Rheum*** 1998; 37: 659-664.
68. Merkel PA, Polisson RP, Chang Y, Skates SJ, Niles JL. Prevalence of anti-neutrophil cytoplasmic antibodies in a large inception cohort of patients with connective tissue disease. ***Ann Intern Med*** 1997; 126: 867-873.
69. Gross WL, Csernok E. Immunodiagnostic and pathophysiologic aspect of antineutrophil cytoplasmic antibodies in vasculitis. ***Curr Opin Rheum*** 1995; 7: 11-19.
70. Wiik A. Rational use of ANCA in the diagnosis of vasculitis. ***Rheum*** 2002; 41: 481-483.
71. Dispenzieri A, Gertz MA, Therneau TM, Kyle RA. Retrospective cohort study of 148 patients with polyclonal gammopathy. ***Mayo Clin Proc*** 2001; 76: 476-484.
72. Hay EM, Freemont AJ, Kay RA, Bernstein RM, Holt PJ, Pumphrey RS. Selective polyclonal increase of immunoglobulin G1 subclass: a link with Sjogren's syndrome. ***Ann Rheum Dis*** 1990; 49: 373-377.
73. Kyle RA, Child JA, Anderson K, Barolgie B, Bataille R, Bensinger R et al. Criteria for the classification of monoclonal gammopathies, multiple myeloma and related disorders: a report of the international myeloma working group. ***Br J Haematol*** 2003; 121:749-757.
74. Kassa SS, Thomas TL, Moutsopoulos HM, Hoover R, Kimberly RP, Budman DR et al. Increased risk of lymphoma in sicca syndrome. ***Ann Intern Med*** 1978; 89: 888-892.
75. Farr M, Tunn EJ, Bacon PA, Smith DH. Hypogammaglobulinaemia and thrombocytopenia associated with sulphasalizine therapy in rheumatoid arthritis. ***Ann Rheum Dis*** 1985; 44: 723-744.
76. Snowden N, Dietch DM, The LS, Hilton RC, Haeney MR. Antibody deficiency associated with gold therapy: natural history and management in 22 patients. ***Ann Rheum Dis*** 1996; 55: 616-621.
77. Mollnes TE, Haga HJ, Brun JG, Nielsen EW, Sjoholm A, Stufeldt G et al. Complement activation in patients with systemic lupus erythematosus without nephritis. ***Rheum*** 1999; 38: 933-940.
78. Davies KA. Complement, immune complexes and systemic lupus erythematosus. ***Br J Rheum*** 1996; 35: 5-23.
79. Liszewski MK, Kahl LE, Atkinson JP. The functional role of complement genes in systemic lupus erythematosus and Sjogren's syndrome. ***Curr Opin Rheumatol*** 1989; 1: 347-352.
80. Brouet JC, Clauvel JP, Danon F. Biological and clinical significance of cryoglobulins. ***Am J Med*** 1974; 57: 775-788.
81. Cacoub P, Fabiani FL, Mussel. Mixed cryoglobulinaemia and hepatitis C virus. ***Am J Med*** 1994; 96: 124-132.
82. Speckett G. Oxford Hand Book of Clinical Immunology. Oxford (UK): Oxford Press; 1999. p. 482-489.