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Autoantibodies in healthy blood donors, rheumatic and autoimmune liver diseases

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Sammanfattning

Autoimmunitet är ett vanligt fenomen där immunsystemet känner igen kroppens egna vävnader. Autoimmunitet kan leda till sjukdom om vävnadsskada uppstår. Autoimmuna sjukdomar drabbar 5–10 % av den globala befolkningen och vid många av dessa kan autoantikroppar påvisas. Autoantikropparna kan detekteras med flera olika metoder. I denna avhandling användes line immunoassay och fluorescensenzym immunoassay för att undersöka förekomsten av autoantikroppar hos blodgivare och olika sjukdomsgrupper. Line immunoassay använder sig av remsor medan fluorescensenzym immunoassay använder sig av brunnar. Remsorna och brunnarna är täckta med proteiner som får reagera med serumprov från patienten. Vid förekomst av autoantikroppar sker antingen ett färgomslag (line immunoassay) eller ljusreaktion (fluorescensenzym immunoassay).

Studie I och II: Med EuroLine -Autoimmune Liver Diseases- (IgG) line immunoassay analyserades förekomsten av autoantikroppar associerade med autoimmuna leversjukdomar hos blodgivare, patienter med autoimmuna leversjukdomar samt SLE patienter. Autoantikroppar detekterades hos flera blodgivare. En sällsynt autoantikropp, anti-LC-1, var vanligare hos blodgivare än hos patienter med autoimmuna leversjukdomar. Trots förekomsten av autoantikroppar sågs inget samband med avvikande levervärden hos blodgivare eller SLE patienter. Genom att höja referensvärden minskade antalet ”falskt positiva” resultat. Dock kunde detta inte åtgärda problemet med anti-LC-1, vilket indikerar problem med LC-1-antigenet där ospecifika reaktioner detekteras. Risken för att utveckla autoimmun leversjukdom bedömdes vara låg hos SLE-patienterna, då ingen av dessa utvecklade autoimmun leversjukdom trots flera års uppföljning. Majoriteten av de positiva fynden med EuroLine immunoassay kunde inte bekräftas med andra metoder, vilket talar för att denna metod är mycket känslig.

Studie III: Med EuroLine Systemic Sclerosis (Nucleoli) Profile (IgG) line immunoassay kunde de sällsynta autoantikropparna anti-Th/To och anti-NOR90 påvisas lika ofta hos blodgivare som hos patienter med systemisk skleros. Majoriteten av de andra autoantikropparna var vanligare hos patienter med systemisk skleros jämfört med blodgivare och andra sjukdomsgrupper. Vissa autoantikroppar var associerade med specifika kliniska manifestationer, däribland njurpåverkan hos SLE patienter. Dessa fynd behöver verifieras.

Studie IV: Autoantikroppar som binder U1-RNP proteinet (anti-U1-RNP) kan påvisas hos SLE patienter. Patienter med anti-U1-RNP kan analyseras vidare för förekomst av autoantikroppar mot proteinet RNP 70kDa (anti-RNP70), men det kliniska värdet av detta är osäkert. I denna studie användes fluorescensenzym immunoassay för analys av anti-U1-RNP positiva prover avseende anti-RNP70 för att utvärdera om det tillförde något av kliniskt värde vid SLE. Förekomst av anti-U1-RNP var associerat med lågt antal av vita blodkroppar och mindre organskada. Analys av anti-RNP70 tillförde dock inte någon ytterligare klinisk information.

Slutsats: Euroline -Autoimmune Liver Diseases- (IgG) och EuroLine Systemic Sclerosis (Nucleoli) Profile (IgG) är verktyg som är av värde vid diagnostik av autoimmuna leversjukdomar och systemisk skleros, men metoderna har hög känslighet vilket kan leda till falskt positiva resultat. Genom att höja referensvärden kan man minska risken för detta. Några sällsynta antikroppar hittades oftare hos blodgivare än hos patienter med de olika sjukdomsgrupperna, vilket antyder potentiella problem med antigenkällan. Subtypning av anti-RNP70 hos SLE patienter med anti-U1-RNP tillförde inte något av kliniskt värde.

Abstract

Autoimmunity is a common phenomenon where the immune system recognises the body's own tissues. Autoimmunity can lead to disease if tissue damage occurs. Autoimmune diseases affect 5–10% of the global population and in many of these autoantibodies can be detected. The autoantibodies can be detected with several different methods. In this thesis, line immunoassays and fluorescence enzyme immunoassay were used to investigate the presence of autoantibodies in blood donors and various disease groups. Line immunoassays use strips while fluorescence enzyme immunoassay uses wells. The strips and wells are coated with proteins that are allowed to react with serum samples from the patient. In the presence of autoantibodies, either a color change (line immunoassay) or a light reaction (fluorescence enzyme immunoassay) occurs.

Study I and II: With the EuroLine -Autoimmune Liver Diseases- (IgG) line immunoassay, the presence of autoantibodies associated with autoimmune liver diseases was analysed in blood donors, patients with autoimmune liver diseases and patients with SLE. Autoantibodies could be detected in several blood donors. A very rare autoantibody, anti-LC-1, was more common in blood donors than in patients with autoimmune liver diseases. Despite the presence of the autoantibodies, no association was seen with abnormal liver values in blood donors or patients with SLE. By raising the cut-off, the number of "false positive" results decreased. However, this could not correct the problem with anti-LC-1, which seems to indicate that there is a problem with the LC-1 antigen so that non-specific reactions are detected. The risk of developing autoimmune liver disease was considered to be low in the SLE patients, as none of these patients developed autoimmune liver disease despite several years of follow-up. Most of the positive findings with the EuroLine immunoassay could not be confirmed with other methods, indicating that this method is very sensitive.

Study III: With the EuroLine Systemic Sclerosis (Nucleoli) Profile (IgG) line immunoassay, the rare autoantibodies anti-Th/To and anti-NOR90 could be detected as frequently in blood donors as in patients with systemic sclerosis. Most of the other autoantibodies were more common in patients with systemic sclerosis compared to blood donors and other disease groups. Some of these autoantibodies were associated with specific clinical manifestations, including renal involvement in patients with SLE. These findings need to be verified.

Study IV: Autoantibodies that bind the U1-RNP protein (anti-U1-RNP) can be detected in patients with SLE. Patients with anti-U1-RNP can be further analysed for the presence of autoantibodies against the protein RNP 70kDa (anti-RNP70). However, the clinical value of further analysis of anti-RNP70 is uncertain. In this study, fluorescence enzyme immunoassay was used to analyse anti-U1-RNP positive samples for anti-RNP70 to evaluate whether it added anything of clinical value in SLE patients. Presence of anti-U1-RNP was associated with low white blood cell counts and less organ damage. However, analysis of anti-RNP70 in patients with SLE did not add any additional clinical information.

Conclusion: Euroline -Autoimmune Liver Diseases- (IgG) and EuroLine Systemic Sclerosis (Nucleoli) Profile (IgG) are tools that are of value in the diagnosis of autoimmune liver diseases and systemic sclerosis, but the methods have high sensitivity which can lead to false positive results. By raising the cut-off, the risk of this can be reduced. Some rare antibodies were found more frequently in blood donors than in patients with the different disease groups, suggesting potential problems with the antigen source.
Subtyping of anti-RNP70 in SLE patients with anti-U1-RNP did not add anything of clinical value.

Abbreviations

AC	Anti-cell antibody
AChR	Acetylcholine receptor
ACR	American College of Rheumatology
ADCC	Antibody-dependent cell-mediated cytotoxicity
AECG	American-European consensus group
AF	Arthritis Foundation
AIH	Autoimmune hepatitis
AILD	Autoimmune liver diseases
ALBIA	Addressable laser bead immunoassay
ALP	Alkaline phosphatase
ALT	Alanine transaminase
AMA	Anti-mitochondrial antibodies
ANA	Anti-nuclear antibodies
APC	Antigen-presenting cell
ASC	Autoantibody Standardisation Committee
AST	Aspartate transaminase
BCOADC	Branched-chain 2-oxoacid dehydrogenase complex
BCR	B-cell receptor
CDC	Centers for Disease Control and Prevention
CENP	Centromere protein
CTD	Connective tissue diseases
CTL	Cytotoxic T lymphocytes
DC	Dendritic cells
dsDNA	Double-stranded DNA
E. coli	Escherichia coli
EASI	European Autoimmune Standardisation Initiative
ELISA	Enzyme linked immunosorbent assay
EULAR/ACR	European Alliance of Associations for Rheumatology/ACR
EuroLine Liver	EuroLine Autoimmune Liver Diseases (IgG)
EuroLine Myopathy	EuroLine Autoimmune Inflammatory Myopathies 16 Ag (IgG)
EuroLine SSc	EuroLine Systemic Sclerosis (Nucleoli) Profile (IgG)
FEIA	Fluorescence enzyme immunoassay
GBM	Glomerular basement membrane
GGT	Gamma-glutamyltransferase
gp210	Glycoprotein 210
HBD	Healthy blood donors
HEp-2	Human epithelial cell line
HLA	Human leukocyte antige
HRP	Horseradish peroxidase
IC	Immune complex
ICAP	International Consensus on Antinuclear Antibody Patterns

ID	Immunodiffusion
IFCC	International Federation of Clinical Chemistry and Laboratory Medicine
IFN-I	Type I interferon
IFN-I	Type I interferons
Ig	Immunoglobulin
IIF	Indirect immunofluorescence
IRMM	Institute for Reference Materials and Measurements
ISG	Interferon stimulated genes
ITGAM	Integrin subunit alpha M
IUIS	International Union of Immunological Societies
LC-1	Liver cytosol 1 antigen
LE	Lupus erythematosus
LIA	Line immunoassay
LKM-1	Liver kidney microsomal 1
LR	Likelihood ratio
MAC	Membrane attack complex
MCTD	Mixed connective tissue disease
MHC	Major histocompatibility complex
MS	Multiple sclerosis
NBT/BCIP	Nitroblue tetrazolium chloride/5-Bromo-4-chloro-3-indolyl phosphate
NK	Natural killer
NPV	Negative predictive value
OGDC	2-oxoglutarate dehydrogenase complex
PAH	Pulmonary arterial hypertension
PBC	Primary biliary cholangitis
pDC	Plasmacytoid dendritic cells
PDC	Pyruvate dehydrogenase complex
PDC	Pyruvate dehydrogenase complex
PE	Phycoerythrin
PML	Promyelocytic leukaemia protein
PPV	Positive predictive value
pSS	Primary Sjögren’s syndrome
PVDF	Polyvinylidene difluoride
RF	Rheumatoid factor
RNAP III	RNA polymerase III
RP	Raynaud's phenomenon
SI	Signal intensity
SLA	Soluble liver antigen
SLA/LP	Soluble liver antigen/liver pancreas antigen
SLE	Systemic lupus erythematosus
SLICC	Systemic Lupus International Collaborating Clinics
SMA	Smooth muscle antibodies
Sp100	Speckled protein 100

SS	Sjögren's syndrome
SSc	Systemic sclerosis
STAT4	Signal transducer and activator of transcription 4
TCR	T-cell receptor
T_h	T helper cell
TLR	Toll-like receptor
TMB	Tetramethylbenzidine
T_{reg}	Regulatory T cell
TSAb	Thyroid stimulating autoantibodies
TSHr	Thyroid-stimulating hormone receptor
UTI	Urinary tract infection
WG-HAT	Working Group on Harmonisation of Autoantibody Tests
WHO	World Health Organization

Introduction

Autoimmune diseases comprise many disorders, ranging from organ-specific to systemic inflammatory conditions. Overall, autoimmune diseases are prevalent and it is estimated that 5-10% of the global population is afflicted by at least one autoimmune disease (1-3). The number of autoimmune diseases is steadily increasing. This is mostly explained by increased knowledge about underlying pathogenic mechanisms, revealing that many "idiopathic" disorders have autoimmune pathogenesis. In many of these conditions, autoantibodies can be detected. These autoantibodies can have pathogenic importance or represent an epiphenomenon. They may be used as diagnostic markers (4).

Autoantibody detection has been used in clinical laboratory routine for many years and the diagnostic value often relies on old techniques that have high specificity but low sensitivity. Indirect immunofluorescence (IIF) and immunodiffusion (ID) belong to the old and robust techniques that have been used for many years (4). However, in recent years, several new techniques with higher sensitivity have been introduced and, in addition, an increasing number of autoantibody specificities have been identified.
Line immunoassay (LIA, also referred to as immunoblot) and enzyme-linked immunosorbent assay (ELISA) have been used for several years and often have high sensitivity but lower specificity (5, 6). These methods are now often replaced by multiplexing methods, such as addressable laser bead immunoassay (ALBIA) techniques, that permit simultaneous and rapid detection of multiple specificities (6, 7). All these techniques have different advantages and disadvantages regarding diagnostic sensitivity and specificity.

Nowadays, the diagnostic trend is to analyse large panels of autoantibodies with sensitive techniques. Altogether, it is a significant step forward to have more tools to identify severe and often chronic diseases. However, the diagnostic value is not comparable with the results obtained with the old techniques. This important aspect is not always considered and is poorly known by clinicians. Thus, the clinical immunologist has an essential role in interpreting autoantibody results. There is therefore a strong need for increased knowledge in this field.

One of the well-known and most frequently requested autoantibody tests is autoantibodies against nuclear antigens*,* i.e.*,* antinuclear antibodies (ANA). ANA constitute a large family of autoantibodies with multiple specificities that are associated with many rheumatologic disorders but can also be found in, e.g., autoimmune liver diseases (8). In addition to ANA, an increasing number of autoantibody specificities against several tissue- and cell-associated antigens have been identified and many of these are associated with specific muscular, renal and neurologic autoimmune diseases (9).

The prevalence of many autoantibodies increases with age, even without signs of disease (10). This is important when interpreting test results since most cut-off values are based on young healthy individuals' sera (11). Studying the prevalence and clinical significance of different autoantibody specificities in a large group of healthy individuals would therefore be

of great interest. This thesis aims to explore this subject in patients with rheumatic diseases and autoimmune liver diseases.

Basic immunology

Protection against infections is crucial for survival and is performed by a wide range of mechanisms in the immune system's innate and adaptive arms (12).

Innate immunity recognises conserved pathogen structures and includes many cell types, such as granulocytes, macrophages, natural killer (NK) cells and dendritic cells (DC). DC and macrophages can present antigens to other cells and are also called professional antigen-presenting cells (APC). Other components are epithelial cells that form barriers and a wide range of plasma and tissue proteins. Innate immunity recognises conserved pathogen structures through a limited number of receptors and is necessary for immediate and general protection against pathogens (12).

The adaptive immunity includes B- and T-cells. B-cells mature in the bone marrow while T-cells mature in the thymus. The selection process for B-cells is less well-known than the maturation and strict selection of T-cells in the thymus. After maturation B- and T-cells migrate to peripheral lymphoid organs (e.g., lymph nodes) and there participate in the surveillance of pathogens. B cells also function as professional APC (12).

B- and T-cells recognise epitopes on proteins and can produce immunological memory (12). B-cells bind soluble antigens expressing conformational epitopes with B-cell receptors (BCR) and, if activated, may differentiate into plasma cells and generate soluble antibodies with the same specificity as the cell-bound BCR. Such free antibodies can bind soluble and surface-bound antigens.
T-cells recognise linear protein antigens that are presented on major histocompatibility complex (MHC) antigens with their T-cell receptors (TCR). MHC, which in humans are denominated human leukocyte antigens (HLA), are expressed on the surface of APC. T cells can differentiate into T helper (T_h) cells or cytotoxic T cells (CTL). T_h cells modulate the immunological response through the production of cytokines. CTL can kill cells using perforins and granzyme or the Fas ligand pathway (12).

The similarity between foreign and self-epitopes and the almost infinite repertoire of antigen-recognising BCR and TCR contribute to the recognition of autoantigens as a normal finding (see Immunologic tolerance below).

Immune responses may be very potent and strict control is imperative to avoid excessive tissue damage. Even though autoantigens are recognised, tolerance to autoantigens is the rule. In autoimmune diseases, tolerance mechanisms are dysregulated (12).

Immunologic tolerance

Immunologic tolerance is the ability of the immune system to avoid destructive attacks of the immune system on self-tissues. Immunologic tolerance can be divided into central and peripheral tolerance (13).

Central tolerance of T-cells occurs in the thymus, removing autoreactive lymphocytes with too high affinity for autoantigens. Here, lymphocytes bind to autoantigens and undergo selection. Too strong binding results in apoptosis (negative selection). Weak binding allows permission to be included in the T-cell repertoire (positive selection) (13).
Less is known about the tolerance processes for B-cells, which are supposed to occur in the bone marrow. Since T-cells are important for activating B-cells, the central tolerance for T-cells is most important (13).

Peripheral tolerance mechanisms suppress autoreactive B- and T-cells through a wide range of mechanisms including regulatory T-cells (T_{reg}), barriers, lack of co-stimulation and cytokines promoting anergy or apoptosis (13).

Autoimmunity

Autoimmunity, the presence of autoreactive B- and T-cells, is common in healthy individuals and is not necessarily linked to autoimmune diseases (1, 12). Examples of autoantibodies that can be detected in individuals without autoimmune diseases include the rheumatoid factor (RF) of IgM isotype, which can develop secondary to tetanus toxoid booster vaccination and anti-troponin I autoantibodies in healthy individuals and patients with cardiac diseases (14, 15). However, when autoimmunity results in autoinflammation, resulting in tissue damage, autoimmune diseases can develop. Factors involved in this development include genetic predisposition and environmental factors leading to deleterious activation of the immune system (13, 16).

Risk factors for developing autoimmune disease

Autoimmunity *per se* is thus not sufficient to develop an autoimmune disease (1, 16). A wide range of factors seem to be involved in triggering autoreactive immune cells and the development of autoimmune disease.

With regard to sex, the prevalence of autoimmune diseases is higher among females than males and hormonal factors might contribute to this difference since estrogen has been shown to upregulate immunological activity (16, 17).

Environmental factors include exposure to infectious agents, vitamin D deficiency, smoking and other lifestyle factors such as diet (18, 19). With regard to infection, the term molecular mimicry is often discussed (18). Molecular mimicry refers to the situation where a foreign agent has antigens resembling autoantigens. When the immune system reacts to the foreign agent, activation of autoreactive B- and T-cells targeting the similar autoantigen may also occur, resulting in an autoimmune and autoinflammatory reaction (18).
Another environmental risk factor is UV radiation that causes cellular damage whereby intracellular autoantigens leak and become accessible to autoreactive lymphocytes (20, 21).

Genetic predisposition includes polymorphism of a wide array of genes. Of these, HLA is often the most important and several HLA types are associated with an increased risk of developing different autoimmune diseases, e.g., HLA DRB1*03:01 for systemic lupus erythematosus (SLE) and HLA DRB1*15:01 for multiple sclerosis (MS) (22).

Autoimmune diseases

Autoimmune diseases are characterised by autoreactive B- and T-cells inducing systemic inflammation and/or organ damage (16). Clinical manifestations vary depending on which tissues are affected and the degree of inflammation and tissue damage. Initial symptoms (e.g., fatigue and fever) are often non-specific, which can cause a delay in diagnosis. Because autoantigens are the primary targets, autoimmune diseases often exhibit a chronic clinical course as long as the antigenic targets persist (16). If the target organ is destroyed and the autoantigen thereby is not exposed anymore, the inflammatory reaction abates. However, the lack of function in the target organ remains, e.g., the destruction of insulin-producing cells in the pancreas of patients with diabetes mellitus type 1 (1, 16).

Autoantibodies can be of pathogenic importance or represent a secondary epiphenomenon that may be used as a diagnostic marker. In some diseases, quantitative levels of autoantibodies (e.g., anti-double stranded DNA (anti-dsDNA)) can be used in follow-up and for prognostic evaluation (23). In many rheumatic diseases, e.g., SLE, primary Sjögren's syndrome (pSS), systemic sclerosis (SSc) and mixed connective tissue (MCTD), ANA can be detected (see below) (23).

Autoimmune disease mechanisms

Autoreactive T_h cells, CTL and B-cells contribute to disease mechanisms in different ways. T_h cells secrete different sets of cytokines that direct the immunological response and CTL can kill autologous cells (24).

Autoreactive B-cells differentiate into plasma cells producing autoantibodies (12). Pathogenic autoantibodies can exert their pathological effects in various ways. They can bind to an autoantigen on a cell and induce antibody-dependent cell-mediated cytotoxicity (ADCC) mediated by NK cells or activate the complement system, leading to the formation of a membrane attack complex (MAC, or the C5b-9 complex) and cell death. Autoantibodies bound to soluble antigens, i.e., immune complexes (IC) can deposit in tissues and activate the complement system. Furthermore, the IC can bind to Fc-receptors on macrophages, allowing for phagocytosis. This occurs in the spleen and the liver. Autoantibodies can also mediate a direct pathologic effect by inhibiting or activating receptors and stimulating cytokine production (25, 26). In myasthenia gravis, autoantibodies inhibit the acetylcholine receptor (AChR) and in Graves' disease, autoantibodies activate the thyroid-stimulating hormone receptor (TSHR) (27).

Type I interferons (IFN-I)

Type I interferons (IFN-I) denote a cytokine family that includes the IFN-α, IFN-β, IFN-ε, IFN-ω and IFN-κ subgroups. They are involved in responses to viral infections and are primarily produced by plasmacytoid dendritic cells (pDC). Besides infections, elevated levels of IFN-I can also be detected in patients with SLE, pSS and SSc (28, 29).
IFN-I production is stimulated by activated pathogen recognition receptors (e.g., toll-like receptor (TLR) 7, 8 and 9) (30). These are activated by nucleic acid-containing IC. Most cells can produce low levels of IFN-I (at least IFN-α and IFN-β). However, pDC are able to produce more significant amounts of IFN-I, especially IFN-α (28).
Secreted IFN-I binds to IFN receptors called IFNAR1 and IFNAR2. The downstream result of this is increased expression of a large number of interferon-stimulated genes (ISG). This is called the IFN-I signature. As a result, IFN-I can modulate the number and function of lymphocytes, DC and monocytes (28).

Brief overview of rheumatic diseases studied in this thesis

See **table 1** for disease-associated antigens (31-35). The antigen autoantibody targets are elaborated on in later sections.

Table 1. Clinical associations of autoantibodies against different antigens

ANA	See table 2
SMA	AIH type I
SLA/LP	AIH type I
LKM-1	AIH type II
LC1	AIH type II
AMA-M2	PBC
BPO (AMA-M2)	PBC
Sp100	PBC
PML	PBC
gp210	PBC
Scl-70	dcSSc
RNAP III 11kDa/155 kDa	dcSSc
Fibrillarin (U3RNP)	dc/lcSSc, SLE
Th/To	lcSSc
CENP-A/B	lcSSc, PBC
Ku	lcSSc myositis overlap, SLE
NOR90	lcSSc, RA, SLE, SS
PMScl 75kDa, PMScl 100kDa	SSc myositis overlap
PDGFR	*Unclear*
U1RNP	MCTD, SLE, lcSSc overlap syndrome
RNP70	MCTD, SLE
Ro52/SSA	SARD, AILD, myositis

AILD, autoimmune liver disease; AIH, autoimmune hepatitis; PBC, primary biliary cholangitis; SARD, systemic autoimmune rheumatic diseases; SSc, systemic sclerosis; dcSSc, diffuse

cutaneous systemic sclerosis; lcSSc, limited cutaneous systemic sclerosis; RA, rheumatoid arthritis; SLE, systemic lupus erythematosus; SS, Sjögren's syndrome; MCTD, mixed connective tissue disease

Systemic lupus erythematosus (SLE)

The annual incidence of SLE in Östergötland, Sweden, is approximately 3 per 100,000 individuals with a prevalence rate of 72 per 100,000 individuals (36).
SLE is a systemic autoimmune disease with unknown etiology and it has a heterogeneous clinical presentation with symptoms related to, e.g., the kidneys, skin, heart and the nervous system (37). Among SLE patients, 19-60% may have elevated liver enzyme levels, which can result from various factors, including infections, medications and concurrent autoimmune liver diseases (38, 39). Data on the prevalence of autoimmune hepatitis (AIH) and primary biliary cholangitis (PBC) associated autoantibodies are lacking.

To our knowledge, only one study has analysed the prevalence of SSc-associated autoantibodies in SLE patients (40). Using EuroLine Systemic Sclerosis (Nucleoli) Profile (IgG) (EuroLine SSc) LIA, this study showed that PM-Scl autoantibodies had the highest prevalence in nucleolar ANA-positive SLE patients (4/28 patients, 14.3%). This has also been evaluated in this thesis.
Anti-U1-RNP is detected in up to 30% of patients with SLE (41).

Some of the risk factors for SLE are female sex, adult age, family history of rheumatic diseases, silica exposure and smoking (42). Some of the genetic polymorphisms in signal transducer and activator of transcription 4 (STAT4), IFN-α and integrin subunit alpha M (ITGAM) are associated with an increased risk of developing SLE (43). HLA alleles DRB1*15:01 and DRB1*03:01 are known HLA risk alleles (22).

Primary Sjögren's syndrome (pSS)

pSS is a systemic autoimmune disease that affects the exocrine glands, causing xerostomia, xerophthalmia and glandular swelling. The prevalence rate of pSS ranges between 10-90 per 100,000 individuals in different parts of Sweden (44).

Systemically, any organ can be involved, including the liver (45). Mild liver involvement can be demonstrated in 5-26% of pSS patients (46). The prevalence rate of PBC and AIH in pSS is 4-9% and 1.7-4%, respectively . The prevalence of anti-mitochondrial antibodies (AMA) varies between 1.6-13% with the IIF technique and 3-27% with ELISA and Western blot methods (46). AMA seem to be a risk factor for future PBC development among pSS patients (47). Furthermore, the prevalence of smooth muscle antibodies (SMA) in pSS has been reported within the range of 39-62%. However, only a very small percentage developed AIH (48, 49).

Risk factors include a family history of autoimmune diseases, female sex, adult age, hepatitis C infection, STAT4 gene polymorphism and IFN-I signature (50). Also, certain HLA alleles, such as DRB1*03:01, DQA1*05:01, and DQB1*02:01 confer increased risk for pSS (51).

Systemic sclerosis (SSc)

In the Swedish population, the prevalence rate of SSc is 30 cases per 100,000 individuals (52). SSc is a systemic autoimmune disease of unknown etiology characterised by multiple organ fibrosis. The affected organs include lungs, gastrointestinal tract, kidneys and blood vessels. Raynaud's phenomenon (RP) is seen in nearly all patients (53). SSc is classified as limited cutaneous or diffuse cutaneous SSc based on the extent of skin involvement (54). SSc-associated autoantibodies are seen in >80% of SSc patients. Different autoantibodies are associated with different clinical phenotypes. It is uncommon for patients to have more than one SSc-associated autoantibody (55).

Risk factors are female sex, age >40 years, family history of scleroderma and silica exposure (56). HLA alleles DRB1*11:04, HLA-DQB1*02:02 and HLA-DPB1*13:01 are associated with an increased SSc risk (57).

Mixed connective tissue disease (MCTD)

According to a Norwegian study, the point prevalence rate of MCTD in 2008 was 3.8 per 100,000 individuals (58). MCTD is a systemic autoimmune disease that presents as an overlap with multiple other rheumatic diseases such as SLE and SSc. It can manifest with an array of symptoms, such as RP and puffy hands. For diagnosis, the presence of anti-U1RNP autoantibodies is required (59, 60). The immunodominant subunit of the U1RNP autoantigen is a 70kDa protein denoted RNP70 (61).

Data on risk factors is lacking except for HLA. HLA DRB1*04:01 is associated with an increased risk of developing MCTD (22).

Brief overview of autoimmune liver diseases (AILD) studied in this thesis

Autoimmune hepatitis (AIH)

AIH is a chronic progressive hepatitis that, according to the autoantibody profile, can be divided into two subtypes (type 1 and 2) (62).
In this thesis, AIH type 1 is studied. The estimated prevalence of AIH type 1 ranges from 12-25 cases per 100,000 individuals (62-64). AIH can present acutely with jaundice and rapid progression to cirrhosis. However, more frequently, it presents as an indolent disease with slightly elevated liver enzymes of the hepatocellular pattern (65).

The serological profile of AIH type 1 includes the presence of ANA, SMA and/or autoantibodies against soluble liver antigen/liver pancreas antigen (anti-SLA/LP) (66). SMA and anti-SLA/LP positivity frequency ranges between 50-85% and 10-30%, respectively, while ANA positivity is seen in 50-75% of the AIH type 1 patients (67, 68).

Risk factors are female sex, age (primarily >40 years) and drugs (statins and anti-TNFα treatment) (69). HLA DRB1*04:05 is a risk allele (22).

AIH type 2 patients are usually children and teenagers and this type is characterised by the presence of anti-liver kidney microsome-1 autoantibodies (anti-LKM-1) and/or anti-liver cytosol-1 autoantibodies (anti-LC-1) (70).

Primary biliary cholangitis (PBC)

The prevalence of PBC is estimated to be between 20-40 cases per 100,000 individuals (71, 72). PBC is characterised by inflammatory destruction of intrahepatic bile ducts, resulting in elevated cholestatic liver enzyme values. Large as well as extrahepatic bile ducts are usually spared. PBC can initially be subclinical with only elevated cholestatic liver enzyme values and, over time, progress to cholestatic disease with portal hypertension (73).

More than 90% of individuals with PBC have detectable AMA, specifically type M2 (68, 74). Additionally, several types of ANA, including anti-speckled protein 100 autoantibodies (anti-Sp100) (AC-6, see anti-nuclear antibodies (ANA)), anti-promyelocytic leukemia protein autoantibodies (anti-PML) (AC-6), and anti-glycoprotein 210 autoantibodies (anti-gp210) gp210 (AC-12), can serve as useful markers. About 50% of PBC patients test positive for ANA (75).

Risk factors are female sex, family history of PBC, age >40 years and past cigarette smoking (76). Infections, such as urinary tract infections (UTI), are also considered risk factors due to molecular mimicry related to the pathogen *Escherichia coli* (E. coli) (77). HLA-DR*0801 and HLA-DQB1*0402 are identified as risk alleles (78).

Anti-nuclear antibodies (ANA)

ANA constitute a family of autoantibodies against a wide range of antigens in the cell nucleus. ANA are associated with many rheumatic diseases. Different rheumatic diseases are associated with different ANA specificities, although there is broad overlap – see **table 2** (35, 79). ANA of IgG isotype have the highest diagnostic significance, whereas ANA of IgA and IgM isotypes have low clinical value and are hence not included in the laboratory routine (79, 80).

Table 2. IIF ANA patterns, antigens and assoicated diseases

ANA pattern	**Antigen association**	**Disease association**
Homogeneous (AC-1)	dsDNA, nucleosomes, histones	SLE, drug-induced lupus, JIA
Speckled (AC-4, 5)	hnRNP, U1RNP, Sm, SS-A/Ro (Ro60), SS-B/La, RNA polymerase III, Mi-2, Ku	MCTD, SLE, SjS, DM, SSc/PM overlap
Fine speckled (AC-4)	SS-A/Ro (Ro60), SS-B/La, Mi-2, TIF1γ, TIF1β, Ku	SjS, SLE, DM, SSc/PM overlap
Large/coarse speckled (AC-5)	hnRNP, U1RNP, Sm, RNA polymerase III	MCTD, SLE, SSc
Centromere (AC-3)	CENP-A/B (C)	Limited cutaneous SSc, PBC
Multiple nuclear dots (AC-6)	Sp100, PML proteins, MJ/NXP-2	PBC, SARD, PM/DM
Nucleolar (AC-8, 9, 10)	PMScl 75kDa, PMScl 100kDa, Th/To, B23/nucleophosmin, U3-snoRNP/fibrillarin, RNA polymerase I, hUBF/NOR-90	SSc, SSc/PM overlap, SjS
Nuclear envelope (AC-11, 12)	Lamins A,B,C, lamin-associated proteins	SLE, SjS, seronegative arthritis, PBC

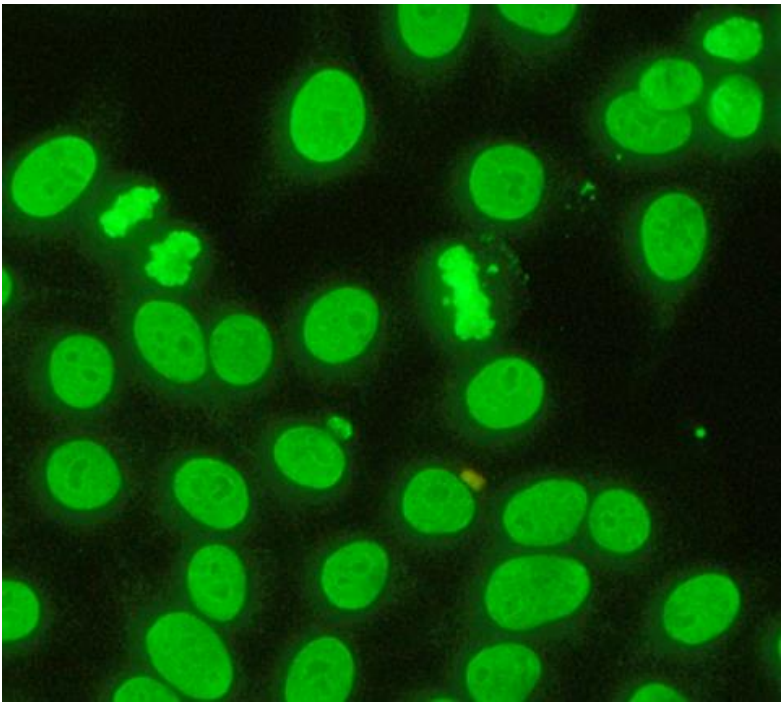
SARD, systemic autoimmune rheumatic diseases; SLE, systemic lupus erythematosus; SjS, Sjögren's syndrome: SSc, systemic sclerosis; MCTD, mixed connective tissue tisease; JIA, juvenile idiopathic arthritis; PM, polymyositis; DM, dermatomyositis; PBC, primary biliary cholangitis

IIF on a human epithelial cell line (HEp-2) is nowadays the golden standard for ANA detection. With this technique, autoantibodies targeting extra-nuclear components can also be detected and the term anti-cell antibodies (AC) has recently been introduced. However, the general denomination “ANA” is still used for historical and practical reasons (35, 81). This thesis generally uses the term ANA to denote positive reactions on HEp-2 cells.

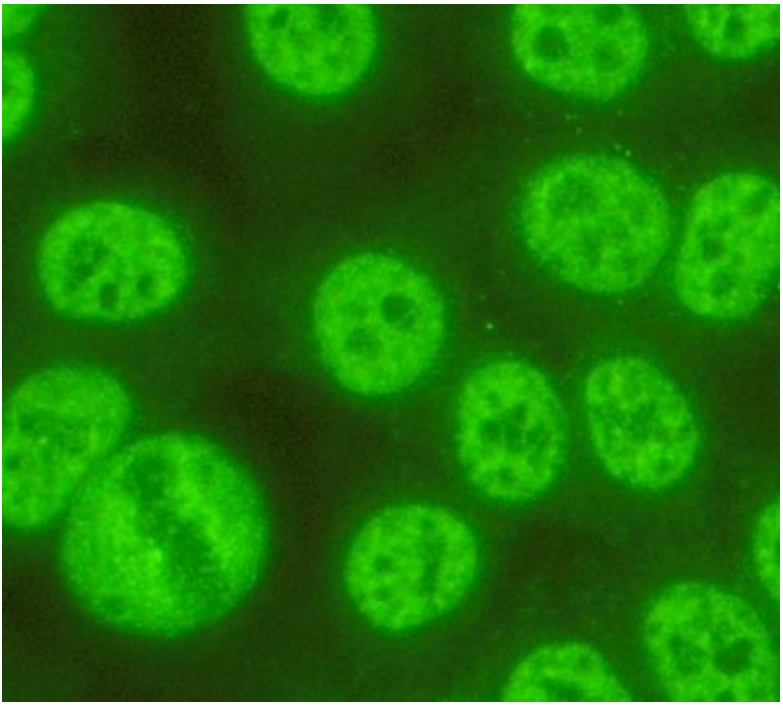
Depending on which antigen the patient's ANA is directed against, different patterns can be identified with IIF and are named accordingly (e.g., homogeneous, speckled, nuclear rim, etc.) – see **figure 1 and table 2** (35). This nomenclature has been used for decades and is still in use but recently the International Consensus on Antinuclear Antibody Patterns (ICAP) introduced the AC-nomenclature that also includes cytoplasmic patterns. The individual patterns are now defined as unique AC numbers; for example, the homogeneous pattern is referred to as AC-1 (35). Several autoantibody specificities can give rise to the same pattern, and therefore IIF HEp-2 needs to be supplemented with other antigen-specific assays to identify the ANA specificity.

For some of the ANA patterns, the ICAP nomenclature uses subtyping. One example is the speckled ANA pattern, where ICAP differentiates between nuclear fine speckled (AC-4) and nuclear large/coarse speckled (AC-5). It can be difficult to visually differentiate the patterns, and computer-based solutions can be a way to standardise the description of the patterns (35).

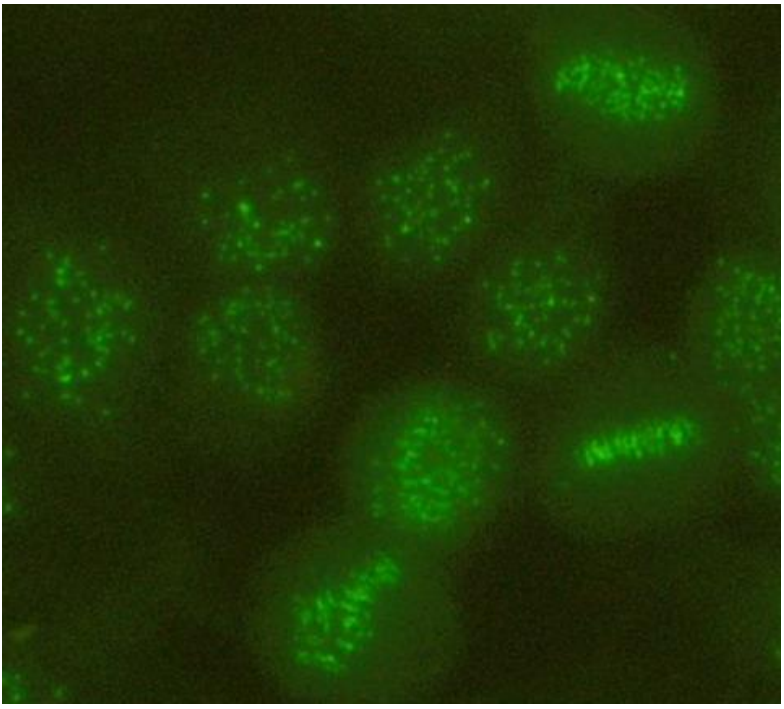
Figure 1. Common ANA patterns



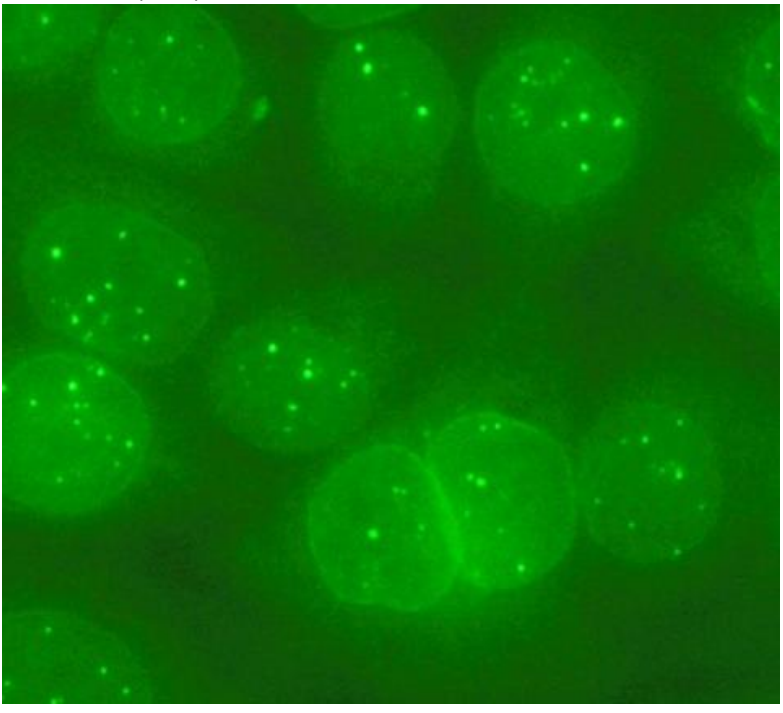
Homogeneous (AC-1)



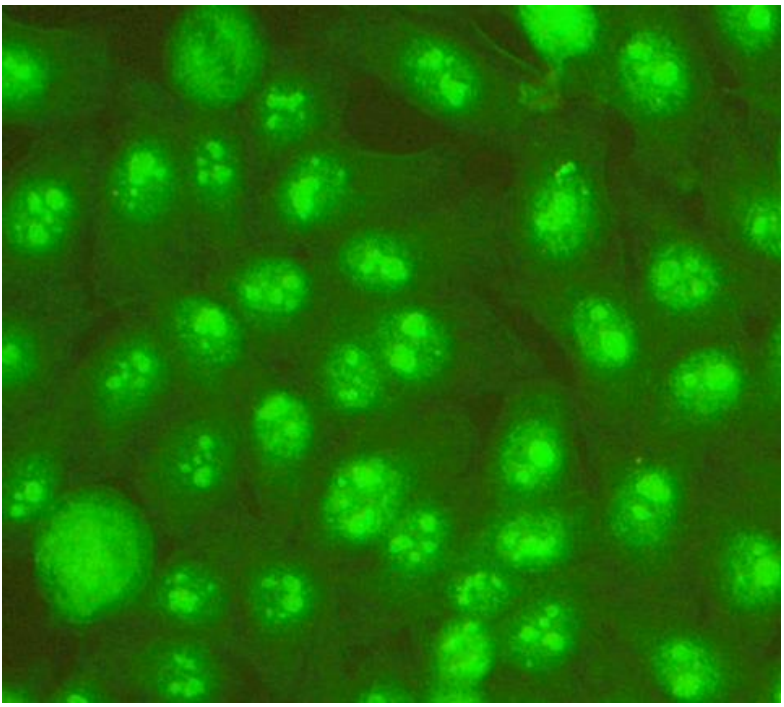
Speckled (AC-4, 5)



Centromere (AC-3)



Multiple nuclear dots (AC-6)



Nucleolar (AC-8, 9, 10)

Autoantibody assay methods

Serological testing for autoantibodies is valuable in the diagnostic workup, follow-up and assessment of the prognosis of rheumatic diseases (16).

Autoantibodies can be detected with techniques that have been used for decades, such as IIF and ID (7). In these assays, autoantibodies react with native antigens in HEp-2 cells or in agarose gel. The diagnostic sensitivity is often lower, but the specificity is higher for these methods compared to newer techniques. The clinical value of results obtained with the older techniques is well-established and validated based on long clinical experience (81). However, the "newer" techniques, such as LIA, ELISA and ALBIA, have been introduced and are now standard methods in clinical laboratories. These methods mostly use recombinant antigens. They have many advantages since they have high diagnostic sensitivity, can be automated and are antigen-specific. The disadvantage is that the experience is limited and the higher sensitivity confers lower specificity (81).
From this, it is evident that results obtained with the old techniques do not always agree with results from the new techniques, and vice versa. This means that the interpretation of the clinical value of the results may be difficult (81).

Regardless of technology, detection of autoantibodies is based on common principles. A relevant antigen source is central. Native, purified or recombinant antigens are often used.

These may expose irrelevant epitopes if denatured or become contaminated with irrelevant antigens (82, 83).
Antigens of interest are coated on a solid phase (wells, strips or beads). The antigens are incubated with patient sera. If the sera contain autoantibodies, these will bind to the antigens. This is followed by a washing step to wash off unbound antibodies. For detection of the autoantibodies, conjugated anti-human IgG antibodies are added. Finally, a readout is performed. In the case of LIA and ELISA, color change is inspected visually or by an instrument (e.g., a scanner) – the higher the color intensity the more autoantibodies does the patient have. In the case of IIF, FEIA or ALBIA, fluorescence is assessed (with a microscope or flow cytometer) – the brighter the more autoantibodies does the patient have (84).
Older techniques, such as ID, include the detection of precipitating IC using soluble native antigens. The precipitated IC form lines in the agarose gel and are assessed visually (84).

Historical techniques

Discoveries related to ANA were made during the 1940s (85). In 1941, SLE was recognised as one of the rheumatic diagnoses (86). Later that decade, in 1948, Malcolm Hargrave and colleagues described a hitherto unknown type of cells called the lupus erythematosus (LE) cells in bone marrow samples of SLE patients (87). LE cells were found to be mature neutrophil granulocytes that had phagocytosed nuclear material from cells ruptured *in vitro*. A similar phenomenon was also discovered *in vivo* in bone marrows from SLE patients, where nuclei were seen to adhere to and were phagocytosed by immature myeloid cells (88). This laid the foundation for multiple discoveries of what became known as ANA (85).

It soon became apparent that LE cells could be found in the peripheral circulation, indicating that LE cells were the result of some unknown primary factor appearing *in vivo* (89). This was apparent from studies where healthy individuals' bone marrow cells were incubated with plasma from SLE patients, leading to the formation of LE cells. The primary factor was found to be a serum factor reacting with nuclear components. In 1957, Holborow incubated LE serum factor with different tissues, including the heart, spleen and kidney and noticed that there were antibodies that bound to these tissues. This was visualised by IIF, where nuclear fluorescence was detected. The same year, findings were published that showed that the LE serum factors were autoantibodies against cell nuclear antigens (89). In the 1950s, an IIF method for detection of these ANA was introduced using rat liver sections as substrate (89). From here, the importance of ANA in clinical diagnostics was initiated.

Various techniques have been used for detecting ANA specificities. During the 1950s-1960s IIF, immunodiffusion and complement fixation methods were developed, followed in the 1970s by Western blot and ELISA, and subsequently by more modern techniques like LIA and ALBIA (85).

Techniques

Below follows descriptions of the laboratory methods used in this project.

Indirect immunofluorescence (IIF) on HEp-2 cells

IIF on HEp-2 cells as antigen source is widely used and still considered the golden standard technique for the detection of ANA. HEp-2 is a human cell line with epithelial cells of laryngeal carcinoma origin (80, 90). They express all native human antigens and have large nuclei, facilitating visual assessment of ANA patterns (81).

IIF HEp-2 analysis was performed with light microscopy using an Olympus BX43 microscope (Olympus, Tokyo, Japan) with a LED diode with light intensity adjusted to 50% of maximal brightness. ANA screening was performed with a 20x objective. HEp-2 cells fixed on slides (ImmunoConcepts, Sacramento, California, USA) were used as antigen substrates. Detection antibodies were FITC conjugated γ-chain specific anti-human IgG antibodies (DAKO, Glostrup, Denmark). Patient sera were diluted 1:800, a dilution yielding 5% positivity (95% specificity) among 752 blood donors analysed in our laboratory, which is in agreement with the international recommendations for assessment of ANA (80).

IIF on fixed rat liver, kidney and stomach sections

IIF on tissue sections from rat liver, kidney and stomach as antigens are used to detect autoantibodies associated with AIH and PBC. SMA are primarily directed against F-actin and these autoantibodies are strongly associated with AIH type 1 (66). The SMA pattern is characterised by fluorescence of smooth muscle cells in blood vessels, gastric submucosa and gastric interglandular actin fibers. Anti-LKM-1 presents as fluorescence in hepatocytes and proximal renal tubules. Anti-LKM-1 is seen in AIH type 2 (68). Fluorescence in parietal cells and both proximal and distal renal tubules indicate the presence of AMA which can be of two types, namely AMA-M2 and AMA-M4. AMA-M2 is a strong diagnostic marker for PBC (91).

IIF microscopy was performed on slides with fixed rat liver, kidney and stomach (NOVA Lite®, Werfen, San Diego, CA, USA). Detection antibodies were FITC-conjugated γ-chain-specific anti-human IgG antibodies (Werfen). An Olympus BX43 microscope was used. For screening, patient sera were diluted 1:400, corresponding to 5% SMA positivity in 200 healthy blood donors analysed in our laboratory.

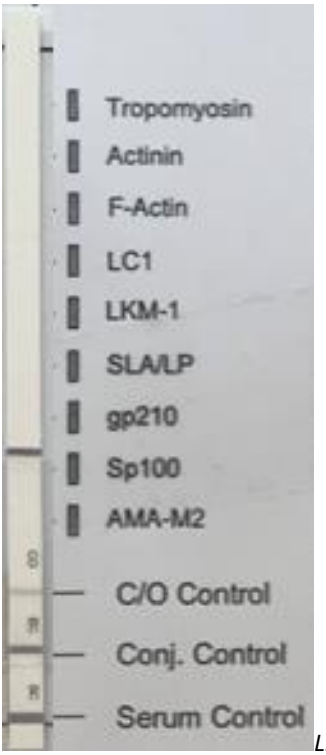
ELISA and fluorescence enzyme immunoassay (FEIA)

ELISA and FEIA are very similar in that they use wells coated with antigens (92). The main difference is that there is a color change with ELISA in the presence of autoantibodies, while FEIA is based on fluorescence. A wide variety of autoantibodies can be detected with these techniques - from AILD-associated autoantibodies to autoantibodies associated with rheumatic diseases (85). FEIA analyses were performed with EliA™ reagents (Phadia 250, Thermo Fisher Scientific, Phadia AB, Uppsala, Sweden).

Line immunoassay (LIA)

LIA involves coating a nitrocellulose or polyvinylidene difluoride (PVDF) membrane with purified or recombinant antigens – see **figure 2**. The antigens are mostly recombinant. After application of antigens, a blocking solution is added to prevent non-specific binding before the commercial reagents are distributed to customers (93).

Figure 2. LIA coated with different controls and antigens associated with AILD. Here anti-Sp100 is detected.



LIA, line immunoassay; AILD, autoimmune liver disease

The membrane strips are incubated with diluted serum samples, followed by the addition of anti-human IgG (Fc) detection antibodies, which can be conjugated with horseradish peroxidase (HRP) or alkaline phosphatase (ALP). Different substrate solutions are added to visualise antigen-antibody interactions depending on the conjugated enzyme. For HRP-conjugated antibodies, tetramethylbenzidine (TMB) is used, and for ALP-conjugated antibodies, nitroblue tetrazolium chloride/5-bromo-4-chloro-3-indolyl phosphate (NBT/BCIP) is used. ALP and HRP convert their corresponding substrates into colored insoluble products, which stain the membrane strips in the corresponding locations. The color intensity correlates to the quantity of antibodies bound up to a certain limit where the staining reaches a plateau. Lastly, evaluation is performed either visually or with scanning densitometry. The latter is the preferred method as it allows a certain degree of quantification of signal intensity (SI) (93).

Compared to ELISA and FEIA, an advantage with LIA is the possibility to analyse multiple autoantibody specificities in parallel. Similar to other solid-phase immunoassays, the drawback is that changes in the recombinant antigens' immunological and chemical properties as part of processing and fixation may induce clinically irrelevant neo-epitopes, lowering the diagnostic specificity and making it difficult to define an optimal cut-off (94-96). A weakness of the studied EuroLine LIA is that the assays do not contain any internal quantitative standard for the individual autoantibody specificities, making it difficult to quantify the SI for the corresponding autoantibody reactivities (97, 98).

Attention to experimental conditions, including room temperature, is crucial. An increase in temperature can affect the results as the enzymatic activity increases, potentially changing the results from negative or inconclusive to weakly positive (94, 99). Furthermore, most commercial solid phase assays do not correct for the background reactivity in individual sera. This sometimes can make it difficult to discern between true positive and false positive reactions (100).

EuroLine SSc and EuroLine Autoimmune Liver Diseases (IgG) (EuroLine Liver) LIA do not have any kit calibrators, but they do have positive control bands that detect if the investigated fluid contains human IgG.
These two kits were used in our studies according to the manufacturer's instructions. The samples were analysed with EuroBlotmaster (Euroimmun AG), and after drying, the strips' SI were evaluated with EuroLineScan (Euroimmun AG).

EuroLine Liver LIA has been used together with a pooled internal control, consisting of autoantibodies against AMA-M2, BPO, Sp100, PML, gp210, LKM-1 and SLA/LP, since 2014 in our laboratory. The mean SI and CV for each of the specificities included in our pooled internal control are shown in Paper II. EuroLine SSc LIA has not yet been adopted in clinical routine, and consequently, we do not have any long-term data on the stability of internal controls.

Addressable laser bead immunoassay (ALBIA)

This multiplex method is based on fluorochrome-labeled microspheres (beads) coated with antigens. Each bead is coated with a specific antigen and can be identified by its quantitative expression of two fluorescent colors. In ALBIA, the detection antibody is conjugated with phycoerythrin (PE), which emits a third fluorescence upon exposure to light (7).
The stronger the fluorescence, the more autoantibodies the patient has against that particular antigen. Data are collected by a highly specialised flow cytometer, allowing quantitation of multiple autoantibody specificities in parallel in a single blood sample (7).

ALBIA assays were performed with FIDIS™ Connective Profile reagents and Solinium software version 1.7.1.0 (Theradiag, Croissy-Beaubourg, France). A Luminex® 200™ instrument (Luminex Corporation, Austin, Texas, USA) was used for analysis.

Discrepancy between autoantibody assays

The agreement between the methods to determine and to quantitate autoantibodies can be poor due to multiple reasons. Manually performed methods can have high inter-operator variability, e.g., visual interpretation of IIF reactions. This can be mitigated by using automated methods. Another factor is different antigen sources (101).

Antigens used in immunoassays are of purified or recombinant origins. The exact sequence, conformation and post-translational modifications of the antigens are vital. Purified antigens often keep the native protein structure, but contamination with irrelevant antigens may occur. The main disadvantages of native antigens are limited quantity and purification challenges. This, in turn, has a negative impact on reproducibility (102).
In contrast, recombinant antigens may expose irrelevant linear epitopes or have some form of alteration (82). However, recombinant antigens have higher levels of purity and can be produced in large quantities. Furthermore, recombinant antigens, especially those synthesised by bacteria, may lack post-translational modifications. For this reason, the antigens are often produced in eukaryotic cells, but this has disadvantages such as extraction difficulties and reduced immunoreactivity (82).

Maintaining the three-dimensional structure of antigens is crucial to avoid false negative reactions (82). False positive reactions can occur when the purification process has not removed contaminants sufficiently or when denaturation has introduced neoepitopes (83).

Modern immunoassays, including LIA, primarily use recombinant antigens with a potential risk of denaturation as part of production or fixation to a solid phase (100). This is highlighted by, e.g., anti-TIF1γ that primarily targets conformational epitopes, causing some LIA to miss this important cancer-associated autoantibody (103). Another example is anti-Mi2, where LIA seems to have a lower sensitivity (104).
The older techniques, such as immunoprecipitation and immune diffusion, use native antigens in soluble form. This approach maintains the antigen's native conformation and is closest to representing the *in vivo* conformation (100).

Other factors include level of expression of antigens and the number of antigens exposed or not exposed. In HEp-2 cells, certain antigens are weakly expressed (e.g., Ro60) and these can often not be detected by IIF. Furthermore, LIA, ELISA, FEIA and ALBIA can only detect a limited number of specificities, while HEp-2 cells can detect far more. HEp-2 cells and LIA strips can be visually assessed, which introduces subjective errors (101). Furthermore, LIA can be interpreted as a dry or wet strip, which can give different results (97).

The choice of detection antibody is of central importance in ANA diagnostics. The detection antibody should specifically detect human IgG (Fc) and not cross-react with other immunoglobulin isotypes. Autoantibodies of IgG isotype are clinically relevant whereas IgA and IgM isotypes are of low clinical value (81).

Cut-off values

This section describes the general principles for the determination of cut-off values. The cut-off value is the numerical value or dilution used to differentiate between positive and negative results.

An appropriate cut-off (i.e., dilution) of IIF HEp-2 ANA corresponds to the 95th percentile of the results of sera from a healthy population. International guidelines recommend a dilution of patient sera of 1:160 or a titer corresponding to the 95th percentile of a healthy population. Every laboratory has to define its suitable screening dilution that depends on the microscope technology and individual interpretation (80, 81). Another factor to consider in terms of clinical relevance of ANA is titration. In this context, a high titer means a stronger association with disease than a low titer.

Cut-offs with ELISA, LIA and FEIA are based on the same principles as above, i.e., the cut-off is set at an arbitrary value that gives a maximum of 5% positive healthy individuals. Since rheumatic diseases are uncommon, one should consider re-evaluating this level of specificity and setting cut-offs based on higher levels of specificity (e.g., 98%).

Different methods have different levels of variation at different autoantibody levels, e.g., EuroLine Autoimmune Inflammatory Myopathies 16 Ag (IgG) (EuroLine Myopathy) LIA has higher variation in reactivities close to the manufacturer's recommended cut-off, making differentiation between healthy and diseased difficult (97). Raising the cut-off might increase the diagnostic specificity, but this also decreases the diagnostic sensitivity. The diagnostic sensitivity and specificity of LIA for the different specificities can vary depending on the cut-off, implying that the same cut-off may not necessarily be applied for all specificities (100).

Manufacturers recommend a cut-off based on their control material, consisting of a limited number of blood donors and disease controls. However, each laboratory needs to verify these cutoffs by analysing its own local group of control sera.
Given that some kits are expensive, laboratories tend to examine a small control material for their cut-offs. These cut-offs are less reliable since the probability that they represent the true cut-off in the population is low due to a larger error margin. To achieve a reliable cut-off, one therefore needs to analyse a larger control material.

Given that different laboratories use different assay reagents, cut-offs based on different populations, employ different analysis instruments and have variably experienced personnel, it is not surprising that a laboratory result of an autoantibody analysis sometimes cannot be confirmed in another laboratory. Some may have modified analysis steps, but other factors, such as temperature, contamination and cross-reactivity can also explain the variation (105).

Sensitivity, specificity, accuracy and concordance

Sensitivity describes an assay's ability to identify diseased individuals and classify these as positive cases. Specificity describes an assay's ability to classify healthy or control individuals as negative cases (106).

There is a trade-off between sensitivity and specificity depending on the cut-off. It is known from experience that, in general, concentrations of autoantibodies are lower in healthy individuals compared to diseased individuals. This means that more healthy controls are classified as positive at low cut-offs, indicating a lower specificity than at high cut-offs. However, some diseased individuals can have low concentrations of autoantibodies, which can only be detected using low cut-offs. In this case, a low cut-off is associated with high sensitivity, but the specificity is low (106).

Accuracy is another term used to describe the performance of an assay in classifying diseased and control individuals with positive and negative results. The more diseased individuals are positive with an assay and the more control individuals are negative with the same assay, the higher accuracy the assay has (106).

Concordance describes the level of agreement between two methods (107).

Predictive values and likelihood ratios (LR)

In addition to sensitivity and specificity, there are also other measures to describe an assay's ability to distinguish between patients and controls.

Positive and negative predictive values (PPV and NPV) indicate how well a test can accurately identify disease and control individuals. The PPV indicates the probability that any patient in the group has the disease if the test result is positive. In contrast, the NPV suggests the probability that any individual in the group is healthy if the test result is negative. While sensitivity and specificity are based only on cohorts of patients and controls, PPV and NPV are based on the prevalence of a disease as well as on diagnostic sensitivity and specificity of the assay (108).

LR is based on sensitivity and specificity (108). Positive LR (LR+) can be described as sensitivity divided by (1-specificity). LR for a test result, together with the pre-test probability for disease of a patient, determines the post-test probability for disease for that particular patient. A positive test result with a high LR+ indicates a higher probability of disease. A negative test result with low negative LR (LR-) reduces the probability of disease and can thus be used to rule out diagnosis (108).

Reference sera, standardisation and harmonisation of autoantibody tests

Early on, when autoantibody diagnostics became available for clinical use, it became apparent that the assays used had issues with variability, meaning that different laboratories

obtained discrepant results. Similarly, results could sometimes not be reproduced by the same laboratory (109).

In the early 1980s, Autoantibody Standardization Committee (ASC) was formed to tackle this issue. ASC was the result of a collaboration between Arthritis Foundation (AF), World Health Organisation (WHO), Centre for Disease Control and Prevention (CDC) and the International Union of Immunological Societies (IUIS). ASC was tasked with selecting monospecific serum samples from patients with rheumatic diseases. These collected reference sera were stored at the CDC laboratory for distribution to laboratories around the world (109).

A reference serum is collected from patients with a verified diagnosis and a defined autoantibody. A reference serum can originate from a single individual or be pooled from many individuals. With the help of reference sera, the methods can be calibrated, which facilitates standardisation and comparison. The idea is to align unitage between different assays measuring the same autoantibody and to align sensitivities and specificities of the different methods in a standardised manner (110).

In 2002, the European Autoimmune Standardisation Initiative (EASI) initiated a project different from ASC. Instead of focusing on reference reagents, EASI focuses on harmonising testing algorithms, data sharing and collaboration between clinicians and laboratory specialists (111-113).

In 2009, the International Federation of Clinical Chemistry and Laboratory Medicine (IFCC) formed the Working Group on Harmonization of Autoantibody Tests (WG-HAT). This group collaborates with the Institute for Reference Materials and Measurements (IRMM) to create new reference materials based on well-defined guidelines (109, 110).
ASC, EASI and WG-HAT now work in parallel with harmonisation of autoantibody diagnostics.

Statistics

Descriptive statistics were presented as means, medians, ranges, counts and percentages. Differences in categorical variables were studied using the $\chi 2$ test or Fisher's exact test when appropriate. Continuous variables, e.g., autoantibody levels in different groups, were compared using Student's t-test or Mann-Whitney's U test. ROC analyses were performed to evaluate different cut-off values. Additionally, concordance, PPV, NPV and accuracy were calculated. Spearman's rank correlation test was utilised to investigate the correlation between autoantibodies and other continuous variables such as IFN-α levels. The significance level for statistical tests was set at a two-tailed p-value <0.05, except in Paper III, where a p-value ≤ 0.01 was considered significant.

The following softwares were used: Real Statistics Resource Pack software release 6.8 copyright (2013–2020) Charles Zaiontz (www.real-statistics.com), Microsoft Excel® version 2018 (Redmond, WA, USA), SPSS statistics software V.27.0 (IBM) and Prism V.9 (GraphPad Software, La Jolla, USA).

Ethical approvals

Following ethical approvals were obtained for each study (diary number):
Paper I: Linköping M75-08/2008* and Uppsala 2006/217/2*
Paper II: Linköping 2017/474-31*, 2018/52-32*, 2020-06412* and 2020-06412**
Paper III: Linköping M75-08/2008*, Linköping 2017/474-31*, Lund 590/2008* and 2020-03287**
Paper IV: Linköping M75–08/2008*, 2017/474–31* and 2020-03287**

*The Regional Ethics Boards
** The Swedish Ethical Review Authority

Aims

This thesis aimed to study the following questions:

- What is the prevalence of AILD-associated autoantibodies in blood donors, pSS and SLE patients using the EuroLine Liver LIA? What are its sensitivities and specificities for the individual autoantibody specificities using cut-offs recommended by the manufacturer? Are the recommended cut-offs optimal or should some of them be adjusted?
- What is the prevalence of SSc-associated autoantibodies in blood donors, pSS, SLE and SSc using the EuroLine SSc LIA?
- What is the prevalence of anti-U1RNP and anti-RNP70 in SLE patients compared to MCTD patients, and is there a difference in clinical association between the two autoantibodies?

PAPER I

Autoantibodies associated with primary biliary cholangitis are common among patients with systemic lupus erythematosus even in the absence of elevated liver enzymes
Ahmad A, Heijke R, Eriksson P, Wirestam L, Kechagias S, Dahle C, Sjöwall C.
Clin Exp Immunol. 2021;203(1):22-31.

In this retrospective study, we evaluated the prevalence of AILD-associated autoantibodies in SLE and pSS patients.

Patients and methods
Stored sera from 280 patients diagnosed with SLE and 114 patients with pSS were analysed. The SLE patients fulfilled ACR-82 criteria and/or the Fries' diagnostic principle (114, 115). The pSS patients fulfilled the American-European consensus group (AECG) criteria (116).

Sampling for SLE patients was performed between 2008-2018 and for pSS patients between 2006-2014. Serum samples were stored at -70^{o}C until analysis. All patients were recruited from the Linköping University Hospital.
8 SLE (2.9%) and 3 pSS (2.6%) patients had been diagnosed with AILD prior to the start of this study and were excluded.

IIF on HEp-2 cells (ImmunoConcepts, Sacramento, CA, USA) was used for the detection of ANA and on rat liver, kidney and stomach sections (Nova Lite, Inova/Werfen, San Diego, CA, USA) for the detection of SMA, AMA and anti-LKM. Gamma-chain specific conjugate for detection of autoantibodies of IgG isotype was used for both IIF assays. In addition, EuroLine Liver LIA (Euroimmun, Lübeck, Germany) was used according to the manufacturer's instructions for detection of AILD-associated autoantibodies and the manufacturer's recommended cut-off was used, i.e. >10 SI. An EuroBlotmaster instrument (Euroimmun AG, Lübeck, Germany) was used to analyse the samples. With this assay autoantibodies against following antigens can be detected: M2 (i.e., E2 subunit of pyruvate dehydrogenase); M2-3E (BPO, i.e., a recombinant fusion protein of the E2-subunits of the three main M2-antigens; branched-chain 2-oxoacid dehydrogenase complex [BCOADC]; pyruvate dehydrogenase complex [PDC]; and 2-oxoglutarate dehydrogenase complex [OGDC]); speckled protein 100 (Sp100); promyelocytic leukemia protein (PML); glycoprotein 210 (gp210); liver kidney microsomal 1 (LKM-1) antigen; liver cytosol 1 (LC-1) antigen; soluble liver antigen/liver pancreas antigen (SLA/LP); and SSA/Ro52. ALP-labeled anti-human IgG (goat) was used.

Liver enzyme values were retrieved from medical records. These included ALP, alanine transaminase (ALT), aspartate transaminase (AST) and gamma-glutamyl transferase (GGT). Pathological liver enzyme patterns were categorised as cholestatic [i.e., elevated ALP ± elevated GGT, AST and/or ALT] or hepatocellular (i.e., elevated ALT ± AST).

Results
In SLE patients without AILD diagnosis (n=272), PBC-associated autoantibodies were detected in 26 (9.6%) patients and AIH-associated autoantibodies in 15 (5.5%) patients with the EuroLine Liver LIA. The most common PBC-associated autoantibody in the SLE patients was AMA-M2 (n=20, 7.4%). None of the AMA-M2 findings could be verified with IIF. SMA (n=12, 4.4%) was the most common AIH-associated autoantibody. In addition, one patient

had serological overlap with both PBC- and AIH-associated autoantibodies (against Sp100, LC-1 and SLA/LP).
Cholestatic liver enzyme patterns were found in two (7.7%) of the patients with PBC-associated autoantibodies. Additionally, hepatocellular liver enzyme patterns were found in another two (7.7%) patients. In patients with AIH-associated autoantibodies, a cholestatic pattern was found in one (6.7%) patient and hepatocellular in three (20%) patients. The patient with serological overlap had normal liver enzyme values.

No difference was found in the prevalence of cholestatic liver enzyme values between the SLE group with PBC-associated autoantibodies and the SLE group without any AILD-associated autoantibodies (p=0.08). Similarly, no difference in the prevalence of hepatocellular liver enzyme values was found between the SLE group with AIH-associated autoantibodies and the SLE group without AILD-associated autoantibodies (p=0.16).

In pSS patients without AILD diagnosis (n=111), PBC-associated autoantibodies were detected in nine (8.1%) patients and AIH-associated autoantibodies in 12 (10.8%) patients. AMA-M2 were the most frequent autoantibodies (n=4, 3.6%). Only one AMA-M2 finding could be verified with IIF. SMA was detected in 11 (9.9%) patients and they all had liver enzyme values within reference limits. In addition, two (1.8%) patients had serological overlap with SMA and AMA-M2, and both had normal liver enzymes.
Of the patients with PBC-associated autoantibodies, one had isolated elevated GGT. All patients with AIH-associated autoantibodies had normal liver enzyme values.

The positive autoantibody findings of the studied subjects were mainly detected with the LIA, and AMA-M2 was the most frequently detected autoantibody with LIA. Only a few findings demonstrated with LIA could be verified with IIF. PBC-associated autoantibodies, detected with the LIA, showed similar prevalence in SLE and pSS patients, but no association with laboratory signs of cholestasis was found. The prevalence of SMA in SLE (4.4%) was comparable to the healthy donor population used to define the assay cut-off corresponding to the 95th percentile.

In the SLE cohort, the median SI among AMA-M2 positive patients was 15.5 (range 11-76), while in the pSS cohort it was 29 (range 13-76). The median SI for anti-BPO was 33.5 (range 12-107) in anti-BPO positive SLE patients. However, in the pSS cohort the median SI for anti-BPO was 81.5 (range 32-127). Four patients in the pSS cohort were anti-Sp100 positive (median SI 45, range 16-70). Only one patient had anti-gp210 (SI 24). No patient was anti-PML positive.
In the SLE cohort, the median SI for anti-Sp100 was 108 (range 19-151) among the autoantibody positive subjects. For anti-PML, the median SI was 28.5 (range 17-40) and for anti-gp210, the median SI was 58.5 (range 11-106).

Even after adjusting the cut-off to >41 SI, as suggested in paper II, the prevalence of AMA-M2 (2.2%) remained higher in SLE compared to healthy individuals (1.1%) – see paper II.

Despite a long follow-up period (mean nine years), none of the subjects in the study were diagnosed with AILD during the follow-up period.

Conclusion

PBC-associated autoantibodies were frequently detected in patients with SLE and pSS using EuroLine Liver LIA. However, only a few of these autoantibodies could be confirmed with IIF, and these findings were associated with laboratory signs of cholestasis in only a few patients.

Since few SLE patients were positive for anti-Sp100, anti-PML or anti-gp210, the above-mentioned SI values are not necessarily reliable. However, for AMA-M2 the SI values seem more reliable since many patients had AMA-M2.
From this, it is apparent that the low SI values of AMA-M2 indicate a high analytical sensitivity of the LIA assay. This highlights that low SI values should not trigger further investigations without a clinically grounded suspicion of AILD. It can be argued that the use of immunosuppressive drugs in these patients may inhibit the development of AILD, further supporting the rarity of AILD in SLE patients, as only a few cases have been reported in the literature (117).

PAPER II

Autoantibodies Associated with Autoimmune Liver Diseases in a Healthy Population: Evaluation of a Commercial Immunoblot Test

Ahmad A, Dahle C, Rönnelid J, Sjöwall C, Kechagias S.
Diagnostics. 2022;12(7):1572.

In this study, we analysed blood donor sera with the EuroLine Liver LIA for AILD-associated autoantibodies. Positive results were also analysed with alternative methods. The alternative methods were EliA™ (FEIA), Alegria® (ELISA) and Liver-9-Line (LIA).

Patients and methods

825 blood donors were included, 403 females (median age 46 years, range 18-71) and 422 males (median age 42 years, range 19-77). The sera were sampled between 2018-2019, immediately aliquoted and stored at -70^{o}C until analysis.

Clinical data and autoantibody test results were retrieved from medical records for 60 patients with verified AILD. Of these patients, 30, 27 and 3 had AIH, PBC and AIH/PBC overlap syndrome, respectively. Patients with AIH fulfilled the simplified criteria according to Hennes et al. (2008), and the PBC patients fulfilled the criteria according to the 2018 PBC guidelines (118, 119).

EuroLine Liver LIA was used to detect AILD-associated autoantibodies, according to the manufacturer's instructions. Positive results for AMA-M2, BPO, gp210, Sp100, LC-1 and SLA/LP were further analysed with EliA™, Alegria® and Liver-9-Line immunoblot. EliA was used to verify AMA-M2 and BPO. Specificities verified with Liver-9-Line were AMA-M2, gp210, Sp100, LC-1 and SLA/LP. Specificities verified with Alegria® were AMA-M2, gp210, Sp100, LC-1 and SLA/LP.

Results

With EuroLine Liver LIA, AILD-associated autoantibodies were detected in 4.4% of the blood donors using the cut-off recommended by the manufacturer. These positive responses were weak and could often not be confirmed by alternative methods. Of the nine AMA-M2 and/or anti-BPO positive EuroLine findings, 1 to 4 (11.1-44.4%) could be verified with the different alternative methods. All three anti-Sp100 positive results could be verified with all the alternative methods. None of the anti-gp210 (n=11) could be verified with any of the alternative methods. Out of the 13 anti-LC-1 positive EuroLine findings, 1 to 2 (7.7%-15.4%) could be verified with the different alternative methods. Anti-SLA/LP (n=1), detected with EuroLine, could be verified with Liver-9-Line assay but not with Alegria®. In patients with established AILD, the positive LIA reactivities were generally stronger.

In the blood donor cohort, autoantibodies against LC-1, gp210 and AMA-M2/anti-BPO were the most commonly detected specificities. The LC-1 specificity differed from the other specificities as the positive responses were stronger in blood donors compared to the AIH group (median 27 and 17 SI, respectively). However, the prevalence of anti-LC-1 was higher in the AIH group compared to the blood donor cohort (6.7% and 1.6%, respectively).

ROC analysis indicated that cut-off values could be raised for multiple specificities to increase diagnostic specificity with retained sensitivity.

Conclusion

Autoantibodies detected by EuroLine Liver LIA are considered to have a high diagnostic specificity for AILD. A weak positive result could, in that sense, be viewed as indicative of a clinical diagnosis. This is, however, contradicted by the relatively high number of blood donors with low positive reactivities for autoantibody specificities presumed to be associated with the rare AILD.

The main concerns highlighted are the low diagnostic specificity and issues related to the LC-1 antigen and/or its antigenic source. Raising the cut-off values did increase the specificity with retained sensitivity, with the LC-1 antigen being an exception. One possible explanation is that the recombinant LC-1 antigen used might express irrelevant epitopes.

PAPER III

Autoantibodies associated with systemic sclerosis in three autoimmune diseases imprinted by type I interferon gene dysregulation: a comparison across SLE, primary Sjögren's syndrome and systemic sclerosis

Andraos R, Ahmad A, Eriksson P, Dahlström Ö, Wirestam L, Dahle C, Hesselstrand R, Bengtsson AA, Jönsen A, Andréasson K, Sjöwall C.
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The study aimed to evaluate the prevalence and clinical correlation of SSc-associated autoantibodies and IFN-α levels in serum from patients with SSc, SLE, pSS, and HBD, respectively.

Patients and methods

Stored sera from patients diagnosed with SLE (n=510), pSS (n=116) and SSc (n=57) were analysed. Sera from 236 HBD served as controls.

SLE patients were classified according to the 1982 ACR and/or the 2012 Systemic Lupus International Collaborating Clinics (SLICC) criteria (115, 120). pSS patients fulfilled the AECG criteria (116). The SSc patients were classified according to the 2013 European Alliance of Associations for Rheumatology/ACR (EULAR/ACR) classification criteria (121).

Sampling was performed between 1982-2022. SLE patient sera were included at Linköping University Hospital and Skåne University Hospital, Sweden. SSc sera were sampled at Skåne University Hospital and pSS sera at Linköping University Hospital.
All sera were stored at -70°C until analysis. Clinical data were retrieved from medical records and self-reported data on RP were available for all groups except pSS.

IIF microscopy on HEp-2 cells was used for the determination of ANA. Levels of IFN-α were analysed with the commercial ELISA kit Human IFN-α (pan-specific) ELISAPRO (Mabtech, Nacka Strand, Sweden), detecting IFN-α subtypes 1/13, 2, 4, 5, 6, 7, 8, 10, 14, 16 and 17.

The commercially available EuroLine SSc LIA was used for the detection of 13 SSc-associated autoantibody specificities. These specificities were: Scl-70, CENP-A, CENP-B, RNA polymerase III (RNAP III) 11kDa, RNAP III 155kDa, fibrillarin (U3-RNP), NOR90, Th/To, PM-Scl75, PM-Scl100, Ku, PDGF-R and Ro52/SSA.

Sera with positive EuroLine results were also analysed with FEIA (EliA™) and BlueDot Scleroderma^{12} IgG immunodot (D-tek, Mons, Belgium). EliA was used to confirm the following specificities: Scl-70, CENP-B, RNA polymerase III (entire complex), fibrillarin and PM-Scl100. BlueDot LIA was used to confirm the following antibody specificities: Scl-70, CENP-A, CENP-B, PM-Scl75, PM-Scl100, Ku, RNAP III (entire complex), U1-RNP, Th/To, fibrillarin, NOR90 and Ro52/SSA.

All analyses of specific antibodies were performed according to the manufacturer's instructions. Determination of ANA with IIF was performed at the individual recruitment hospitals according to the laboratory routine using a cut-off corresponding to the 95th percentile of a healthy population (n=752) and a gamma-chain specific anti-IgG-conjugate.

Results

Percentages of individuals positive for any of the EuroLine autoantibodies in each cohort were 86% (SSc), 26% (pSS), 25.3% (SLE) and 25.4% (HBD).

In SSc patients, autoantibodies against Scl-70, CENP-A, CENP-B, RNAP III 11kDa, RNAP III 155kDa, PM-Scl75 and PM-Scl100 were significantly more prevalent than in the combined control group consisting of SLE, pSS and HBD cohorts. No such significance was found for autoantibodies against Ku, fibrillarin, NOR90, Th/To, and PDGFR. Gender-based analysis did not reveal any differences in the autoantibody frequencies between women and men. Among SSc patients, 21.1% had a single specificity, 66.7% had two or more specificities, and 12.3% tested negative for all SSc-associated antibodies. The most common ANA pattern was AC-4 (29.8%) followed by AC-3 (21.1%). 17.5% of the SSc patients had a nucleolar AC-8/9/10 ANA pattern, and most of these were anti-PMScl75 and/or anti-PMScl100 positive.

In SLE patients, anti-Ro52/SSA (38.2%), anti-CENP-A (6.7%) and anti-Ku (5.7%) were the most common autoantibody specificities. In the pSS cohort, anti-Ro52/SSA (81%), anti-CENP-A (10.3%) and anti-CENP-B (9.5%) were the most common specificities. In the HBD cohort, anti-PMScl75 (6.8%), anti-Th/To (5.9%), and anti-Ku (4.2%) were the most common specificities.

Among positive autoantibody reactivities the SI values were highest in the SSc group, except for anti-Ku where SI values were highest in the pSS cohort. Anti-CENP-A and anti-CENP-B SI values were comparably high in the SSc and pSS cohorts. Fibrillarin, NOR90, and Th/To exhibited similarly low SI values across all the cohorts.

A majority (80-90%) of the positive EuroLine SSc LIA findings in the SSc cohort could be verified with BlueDot and EliA. In contrast, EuroLine SSc LIA findings in the SLE, pSS, and HBD cohorts, excluding anti-Ro52/SSA, had relatively low confirmation rates with BlueDot LIA (18%) and EliA (19%) methods. The agreement between the methods was highest for high SI values.

Due to a lack of sera, IFN-α levels could not be determined in the SSc cohort. The levels of IFN-α were higher among pSS than in SLE patients and correlated positively with the number of SSc-associated autoantibodies ($r=0.29$, $p<0.0001$). Anti-Ro52/SSA showed the strongest positive association with IFN-α levels ($p<0.0001$).

RP data was not available for all individuals in each cohort and was lacking for the whole pSS cohort. When combining all the available RP data from SSc, SLE and HBD cohorts (n=711) significant associations between RP and autoantibodies against Scl-70, CENP-B, RNAP III 11kDa, RNAP III 155kDa, and PM-Scl100 were found. Anti-Ro52/SSA exhibited an inverse correlation with RP.

Autoantibodies against Scl70, RNAP III 11kDa and 155kDa were also associated with pulmonary fibrosis and SSc of diffuse type. SSc of limited type was associated with autoantibodies against CENP-A and CENP-B. No association between SSc-associated autoantibodies and arthritis was found among the SSc patients.

In SLE, anti-CENP-A was associated with fulfillment of the "immunological disorder" criterion in the 1982 classification criteria for SLE. Furthermore, anti-CENP-B was associated with serositis and anti-Ku with lupus nephritis.

Using the combined SLE, pSS and HBD cohorts as the control group, accuracy and specificity of EuroLine SSc LIA were calculated with SSc as outcome. Accuracy and specificity were generally high (~95%) but the sensitivity was low, with the highest sensitivity being 26% (for RNAP III 11kDa). In contrast, IIF ANA had low specificity (49%) but significantly higher sensitivity (96%).

Conclusion

EuroLine SSc LIA often detected SSc-associated autoantibodies in pSS and SLE patients. However, many of these results were weakly positive and could not be confirmed with alternative methods. In contrast, autoantibody findings in the SSc-cohort were often strong and could also be confirmed with alternative methods. This shows that the manufacturer's cut-off needs to be adjusted to increase the diagnostic specificity. Interestingly, several individuals in the SLE and HBD cohorts were positive for the rare autoantibody specificities anti-Th/To and anti-NOR90, raising questions about the antigenic source and the clinical usefulness of this part of the currently available assay.

In contrast to previous reports, patients with SSc often tested positive for multiple autoantibodies (31, 122). Part of this can be explained by the fact that anti-CENP-A and anti-CENP-B were counted as two different specificities in our study, while in some studies they are counted as one (123, 124). The same applies to the RNAP III 11kDa and 155kDa specificities, as well as the anti-PM-Scl100 and anti-PM-Scl75k specificities (125). Despite counting anti-CENP-A and anti-CENP-B as one specificity and also applying the same approach to the two RNAP III and PM-Scl specificities, 14 samples were still found to be positive for at least two autoantibodies. This included, amongst others, positivity for autoantibodies against Scl-70, CENP, RNAP III, fibrillarin, Th/To and Ku. In addition, most patients with multiple specificities were positive for anti-Ro52, which may coexist with SSc-associated autoantibodies. Anti-Scl-70, anti-RNAP III 11kDa/155kDa and anti-CENP-A/B were all mutually exclusive in the SSc cohort, which is consistent with the prior studies (31, 126, 127).
However, our results are consistent with other studies that have used the EuroLine SSc LIA, which is clearly a very analytically sensitive method (124, 128). The previous claim of mutual exclusivity was probably due to the lower sensitivity of the older methods (127, 129).

The presence of SSc-associated autoantibodies was linked to higher IFN-α levels, consistent with previous observations. The study also identified associations between SSc-associated autoantibodies and organ involvement, such as pulmonary fibrosis, RP and different SSc subtypes. These findings were in line with previous studies. With regard to SLE patients, the association between anti-Ku and lupus nephritis confirmed the results from a prior study (130). However, the clinical associations of anti-CENP-A and anti-CENP-B, i.e., serositis and immunological disorder according to 1982 ACR criteria, are new and need to be confirmed.

PAPER IV

Doubtful Clinical Value of Subtyping Anti-U1-RNP Antibodies Regarding the RNP-70 kDa Antigen in Sera of Patients with Systemic Lupus Erythematosus
Ahmad A, Brylid A, Dahle C, Saleh M, Dahlström Ö, Enocsson H, Sjöwall C.
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In this retrospective study, we evaluated whether the detection of autoantibodies against RNP-70k Da, a sub-specificity of U1-RNP, added any clinical value in individuals with SLE, pSS and MCTD.

Patients and methods
Stored sera from 114 patients diagnosed with SLE (92.1% females, mean age 40, range 19-76), 54 patients with pSS (96.3% females, mean age 62, range 25-83) and 12 patients with MCTD (all females, mean age 34, range 14-54) were included. Sera from 128 HBD served as controls (89.1% females, mean age 43, range 20-67).

All the SLE patients met 1982 ACR and/or SLICC classification criteria (115, 120). The pSS patients fulfilled the AECG criteria and the MCTD patients fulfilled the Alarcón-Segovia diagnostic criteria (116, 131).

53 SLE patients (46%) tested positive for anti-U1-RNP with the clinical routine assays ALBIA (FIDIS™ Connective profile; Theradiag, Croissy-Beaubourg, France) and/or EuroLine LIA (Euroline Anti-ENA ProfilePlus-1 (IgG); Euroimmun AG, Lübeck, Germany). All HBD were anti-U1-RNP negative with ALBIA, whereas all MCTD patients were anti-U1-RNP positive with ALBIA and/or EuroLine Anti-ENA LIA. No previous anti-U1-RNP data was available for the pSS cohort.

Sera were sampled between 2008-2018 (SLE), 2006-2014 (pSS), 2009-2022 (MCTD) and 2018-2019 (HBD) at the University Hospital Linköping, Sweden. Sera were stored in aliquots at -70˚ C until use.
For this study, all sera were analysed with the EliA™ U1-RNP and EliA™ RNP70 (both FEIA) according to the manufacturer's instructions.

Clinical data were retrieved from medical records. HBD reported symptoms in a health questionnaire formulated by a rheumatologist.

Results
In the SLE cohort, anti-U1-RNP was detected in 34 (30%) and anti-RNP70 in 21 (18%) of the patients. In the pSS cohort, only one patient was positive and had both anti-U1RNP and anti-RNP70 autoantibodies. Among the MCTD patients, 10 (83%) were anti-U1-RNP positive and 9 (75%) were also anti-RNP70 positive. All anti-RNP70 positive sera in all cohorts were also positive for anti-U1-RNP. All HBD sera were negative for both anti-U1-RNP and anti-RNP-70kDa.
The co-occurrence of anti-U1-RNP and anti-RNP70 was predominantly noticed in samples from patients diagnosed with either SLE or MCTD.

In the SLE cohort, no clinical or laboratory differences were found between patients that were anti-U1-RNP positive with or without anti-RNP70. Comparison between anti-U1-RNP positive and anti-U1-RNP negative SLE patients showed that the anti-U1-RNP positive patients more often had hematologic involvement, RP and less damage accrual, i.e., a clinical phenotype resembling MCTD.

Conclusion

We could not find any clinical support for subtyping for the occurrence of anti-RNP70 among anti-U1-RNP positive SLE patients when evaluated with FEIA. Anti-U1-RNP and anti-RNP70 antibodies are rarely detected in HBD and pSS patients.
A limitation of our study was the small number of individuals with MCTD.

Concluding remarks

The main focus of this thesis was to evaluate the prevalence and clinical significance of autoantibodies associated with AILD and SSc in a large group of blood donors and disease cohorts. In papers I and II, we used EuroLine Liver LIA and in paper III, EuroLine SSc LIA. Blood donor sera with positive findings were also analysed with alternative methods (i.e., IIF, dot blot, ELISA, and FEIA), but most positive findings obtained with EuroLine assays could not be confirmed. In paper IV, we studied the prevalence and clinical correlation of anti-U1RNP and anti-RNP70 in disease and blood donor cohorts. In this study, we used FEIA to follow up on positive findings previously obtained with ALBIA and EuroLine anti-ENA LIA. Most positive ALBIA and LIA findings could not be verified with FEIA.

EuroLine Liver LIA is widely used and is a sensitive tool in detecting autoantibodies associated with AILD. This LIA is used by specialists as well as non-specialists as part of diagnostic workup in patients with elevated liver enzyme values. However, elevated liver enzyme values are frequent findings in the general population (10-21.7%) and are most often due to other causes than the rare AILD (132). The test is sensitive but not 100% specific and should therefore not be used in early stages in evaluating pathological liver enzyme values unless there is a clear clinical suspicion of AILD.

The same discussion can also be applied to EuroLine SSc LIA and its relation to RP. The prevalence of RP in the general population is 5-15% and it is most often not associated with SSc, which has a prevalence of 18.9 per 100,000 individuals (133-135). In study III, we found that 25.4% of blood donors, with or without RP, had SSc-specific autoantibodies. This shows that screening for SSc with the EuroLine SSc LIA among patients with RP confers a risk of obtaining many false positives unless other clinical symptoms indicating SSc also exist.

AILD and SSc are examples of rare diseases, meaning that they are uncommon in a primary healthcare setting. If analytically sensitive but diagnostically less specific autoantibody tests are used without a reasonable clinical suspicion of the target diagnosis, there is a clear risk of obtaining many false positive results. These false positive results risk conferring unnecessary worries, follow-up investigations and costs.
However, in a specialist clinic the prevalence of AILD and SSc is higher. Analysis of patients in these selected cohorts at the specialist clinic can confer a higher predictive value of a positive test result than in the primary healthcare setting. This means that EuroLine Liver LIA and SSc LIA are more important for improving diagnostic workups at specialist clinics than in primary care settings.

Given the variability between assays, it is not surprising that results may be divergent. This issue highlights the need for international calibrators, standardisation and harmonisation. The purpose of standardisation is to attain numerically uniform results between different methods. Harmonisation is the process of making results from different methods compatible with each other, i.e., the purpose is not to obtain numerical uniformity (136).

Approaches to standardisation include the use of reference detection methods (also called golden standard methods) and reference sera.

In the case of AILD-associated autoantibodies, the reference detection methods are IIF on rat tissue sections and HEp-2 cells. A significant disadvantage with IIF is that the technique's analytical specificity and sensitivity for some autoantibody specificities are low. However, the higher analytical sensitivity of more modern antigen-specific methods might be on the other extreme side and may be too high. Using validated reference sera, an assay's sensitivity can be tuned and a comparison between new methods and reference detection methods can be made. With time and experience, the new method's sensitivity and specificity may be fine-tuned.

For antigenic specificities that are relatively recently identified, reference detection methods are lacking and the experience is limited and often based on research results in small cohorts. Some antigens that lack such reference methods include Th/To and NOR90. Using the reference detection method approach can mitigate standardisation issues, but the method selection for new antigens takes time. Therefore, it is primarily used for well-established antigenic specificities.

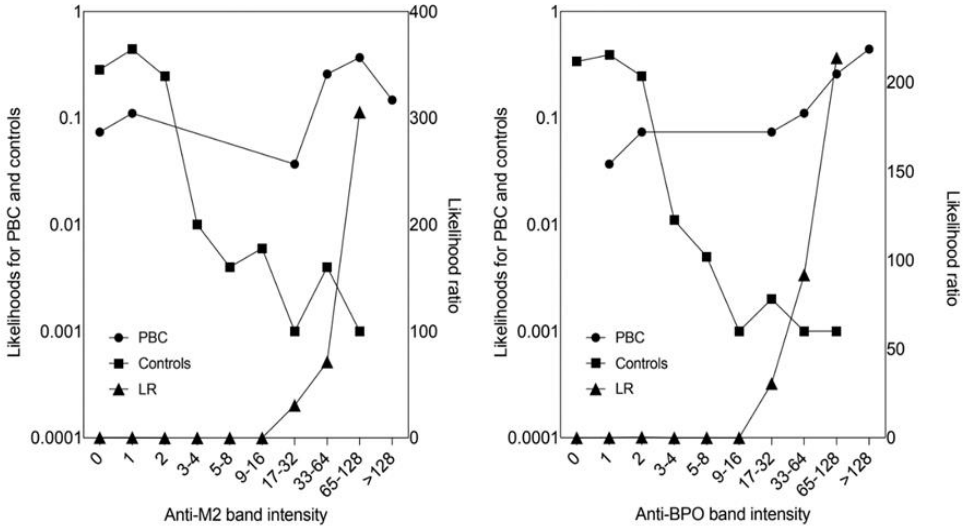
Another important tool in standardisation processes is to use reference sera. Standardisation, by using reference sera, presupposes that autoantibodies target the same epitopes and have uniform avidity. In reality, epitope specificity, avidity, and IgG subclass distribution often differ between serum samples obtained from different patients. Furthermore, reference sera are lacking for many autoantibody specificities.

Hypothetically, using the NIBSC reference serum 67/183 for AMA should make it possible to standardise methods. However, unfortunately, it is highly unlikely that standardisation can be achieved with reference detection methods and reference sera. This is exemplified by a comparison of RF assays that, despite being calibrated against the same international reference serum, still differed up to 15 times between their cut-off values (137).

An alternative to standardisation is harmonisation, which can be achieved with LR based on Bayesian statistics (138). Each patient has a certain setup of clinical signs and symptoms conferring a certain pre-test probability of disease. By means of that individual pre-test probability and the LR of a diagnostic assay, the post-test probability for disease can be calculated for that particular patient (139). With the help of LR, it is possible to assess the clinical relevance of a result and compare different methods regardless of differences in unitage. Furthermore, LR can be calculated for different intervals of quantitative results. Such interval-specific LRs can be compared between different assays for the same autoantibody (140, 141). The calculation of LRs should then be performed by using the same group of patients with the target diagnosis and the same group of controls. Optimally, disease controls with a clinical phenotype similar to patients with the target diagnosis should be analysed.

An example of interval-specific LR+ calculations for AMA-M2 and anti-BPO based on my data on PBC patients and healthy controls in paper II is shown in **figure 3**. Herein, we see that LR+ is low at the manufacturer's recommended cut-off. However, raising the cut-off to >40 SI raises the LR to 70 for AMA-M2 and nearly 100 for anti-BPO. Raising the cut-off even higher does raise the LR+ further, but in that case, to the detriment of sensitivity.

Figure 3. LR calculated for different cut-off intervals. Here AMA-M2 and BPO are shown.



LR, likelihood ratio

We do not fully understand how EuroLine Liver LIA cut-off has been defined and why the same cut-off can be used for all specificities. According to Euroimmun, the antigen's concentration is adjusted so that at the defined cut-off, a sufficiently high diagnostic sensitivity and specificity of the product is achieved (personal communication, Anthonina Ott). This shows that transparency is needed between the manufacturer and customer in order to be able to understand and interpret the test results. We believe this dialogue can be improved.

Interestingly, we found more positive results among blood donors than disease control groups regarding the rare specificities Th/To, NOR90 on EuroLine SSc LIA and LC-1 on EuroLine Liver LIA. This has been communicated with the company and while working on this thesis we have been informed that we will obtain new improved versions of EuroLine LIA for LC-1 and Th/To during 2024 for evaluation.

Our methods detected autoantibodies not only in diseased individuals but also in healthy blood donors. The question is: do certain qualitative characteristics distinguish autoantibodies detected in healthy and diseased populations? Here, thyroid-stimulating autoantibodies (TSAb) come to mind. Through epitope mapping, it has been shown that patients with Graves' disease have TSAb that bind certain epitopes. It has also been shown

that most healthy individuals that have not developed Graves' disease and have TSAb bind other epitopes (142). The same principle should apply to other autoantibodies, e.g., do blood donors and diseased individuals differ in epitope binding? Epitope mapping studies have gained interest and we hope that by defining epitopes we can differentiate between the epitope profiles among diseased and healthy individuals. Building upon this, one can question whether certain epitopes or epitope profiles are associated with some phenotypes, symptoms or risks.

In the U1RNP manuscript, the presence of the anti-U1RNP and anti-RNP70 autoantibodies was related to clinical symptoms. However, in reality it is not as simple as an autoantibody being linked to (or even causing) a particular set of symptoms. An alternative might be to correlate a patient's complete autoantibody profile to symptoms. This then takes into account the combined clinical associations of several autoantibodies and provides a more complete picture of a patient's symptoms and disease phenotype. One way to study this could be with cluster analysis where you study the autoantibody profiles and the detection method used, gender and age (143).

Autoantibody diagnostics is now readily available and often performed with automated instruments. Previously, the clinical immunological laboratories obtained a limited number of samples from clinicians specialised in autoimmune diseases. In that setting, the prevalence of patients with the target diagnosis in the specialist's patient stock and the pre-test probability was high. This situation has now changed, with an increasing number of analyses being requested from non-specialist physicians. In today's situation, the prevalence of the target disease in the investigated population is often much lower, as is the pre-test probability. We are increasingly challenged by a lack of standardisation of methods and limited data regarding the clinical relevance of concurrently occurring autoantibodies. Transparency between customers and manufacturers and collaboration between laboratories and clinicians for studies of larger cohorts of patients and in relevant control groups can be the initial steps to mitigate these problems.

Future perspectives

To approach the question of whether incidental findings of AILD-associated autoantibodies detected with LIA among healthy blood donors might represent sub-clinical disease or very early stages of the disease, we plan follow-up investigations. Five years after inclusion, the plan is to resample the blood donors with positive AILD autoantibody findings at inclusion and to compare them with age- and sex-matched autoantibody-negative controls from the same blood donor cohort. A review of medical records will also be performed. The sera will be analysed with the same detection methods that were used at recruitment, i.e., IIF on HEp-2 cells, IIF on rat tissue, FEIA, EuroLine Liver, SSc and Myopathy LIAs. We will also analyse liver enzymes and the participants will be asked to answer the same questionnaire regarding symptoms as they did at inclusion.

Our findings of positive results in a large cohort of blood donors lacking signs of disease highlight the need to adjust the manufacturer's recommended cut-offs for several autoantibody specificities. Together with our hepatologist, we have evaluated the ROC curves for PBC-associated autoantibodies detected with EuroLine Liver LIA. Our discussions have resulted in an agreement that the cut-off for AMA-M2 and BPO should be raised, which will soon be implemented.

We are not aware of any other clinical routine laboratory that has performed analyses of as big reference materials as we have for defining optimal cut-off values. Hopefully, our results can also be useful for other laboratories. We plan to initiate comparative studies to evaluate if our recommendations can also be applied to other laboratories using the same methodology. In addition, the use of our results as well as reference materials may be helpful to design national guidelines. This issue will be discussed among the experts who are members of the advisory group of Clinical immunology in Equalis, the Swedish provider of external quality assessment schemes. This advisory group also works to produce national guidelines regarding clinical immunology tests.

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Sjöwall, Christoffer

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Papers

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