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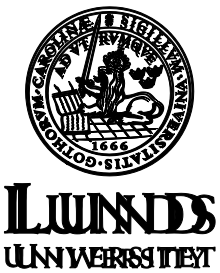
Studies on anti-dsDNA antibodies and other potential biomarkers in Systemic Lupus Erythematosus

Michele Compagno



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Cover image:

The double helix spiral stairway, one upstream and one downstream, at Vatican Museums, Vatican City State, designed by the Italian architect Giuseppe Momo in 1932, long before the double helix structure of DNA was discovered.

It resembles the different steps, upstream and downstream, in the pathogenic stairway leading to Systemic Lupus Erythematosus, including the antibodies to double stranded (helix) DNA.

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"That was a memorable day to me, for it made great changes in me. But it is the same with any life. Imagine one selected day struck out of it, and think how different its course would have been. Pause you who read this, and think for a moment of the long chain of iron or gold, of thorns or flowers, that would never have bound you, but for the formation of the first link on one memorable day."

Charles Dickens

Introduction

Preface

This thesis is the result of my long journey into the field of clinical research in Rheumatology. I started to become interested in Rheumatology when I attended the first lecture on rheumatic diseases at Palermo University in 1992. The one who would have become my very first mentor and tutor, Alfio Pappalardo, talked about many mysterious diseases, affecting a large amount of the population, a few effective treatments, a lot to be done to discover the causes and the underlying mechanisms. Before that I was quite sure that I should become a pediatrician or an ophthalmologist! Ten years later, I started my career as a specialist at the Rheumatology department in Lund, Sweden. I had just moved to Lund to join my wife and our first child, after completing my trainee period at the Rheumatology department in Bari, Italy. When I took my graduation exam in 2001, I presented the results of a research project that I had performed with my first Swedish tutors, Dick Heinegård and Tore Saxne. The project regarded the diagnostic and prognostic role of human Osteopontin in rheumatic diseases. Just Dick Heinegård was the one who first described this protein and suggested it to be named "Osteopontin". Fourteen years (and three more children) later never could I have imagined that Osteopontin would have been a crucial part of my doctoral thesis. I regret that Dick can never read it and criticize it!

During my PhD studies, I enjoyed the learning from several teachers and the tutorship of other colleagues, such as my main supervisor Anders Bengtsson and my two co-supervisors, Gunnar Sturfelt and Andreas Jönsen. To develop the content of the four original papers included in this thesis has been very exciting and challenging. I started the enrollment of the first patients to my research project in November 2004. This journey would have been much longer without the helping contribution of my supervisors, all the other collaborators belonging to the SLE research group in Lund and all the coauthors of the scientific papers.

I hope the persons who read this thesis would appreciate my effort to simplify their appraisal and interpretation of the original papers. For the purpose, I have divided the thesis in three main sections: the first concerns the main features of SLE; the second section is a simplified description of the human immune system,

followed by the abnormalities found in SLE patients; the third section concerns general concepts about biomarkers, followed by a description of the most relevant biomarkers in SLE.

I am afraid I will never be awarded for the groundbreaking scientific content of this thesis, but I am very pleased with the results I have achieved with my coworkers, anyway. This is a "fruit" which has been growing and getting mature during several years, without the help of any dangerous products or forbidden treatments. I have grown myself, from young, enthusiastic medical doctor to more mature and older curious researcher, with a deeper knowledge of the field, a better ability to perform clinical research, new ideas for the future and still…a lot of enthusiasm!

I hope it is contagious and I will transmit it to some of the readers.

List of papers included in the thesis

I. Compagno M; Jacobsen S; Rekvig OP; Truedsson L; Heegaard NH; Nossent J; Jönsen A; Jacobsen RS; Eilertsen GØ; Sturfelt G; Bengtsson AA – **Low diagnostic and predictive value of anti–dsDNA antibodies in unselected patients with recent onset of rheumatic symptoms: results from a long–term follow–up Scandinavian multicentre study**. Scand J Rheumatol, 2013; Vol. 42 (4), pp. 311–6.

II. Compagno M; Rekvig OP; Bengtsson AA; Sturfelt G; Heegaard NH; Jönsen A; Jacobsen RS; Eilertsen GØ; Fenton CG; Truedsson L; Nossent JC; Jacobsen S – **Clinical phenotype associations with various types of anti–dsDNA antibodies in patients with recent onset of rheumatic symptoms. Results from a multicentre observational study.** Lupus Sci Med, 2014 Apr 01; Vol. 1 (1), pp. e000007.

III. Compagno M; Gullstrand B; Jacobsen S; Eilertsen GØ; Nilsson JÅ; Lood C; Jönsen A; Truedsson L; Sturfelt G; Bengtsson AA - **Testing serum-mediated phagocytosis of necrotic material by polymorphonuclear leukocytes predicts clinical manifestations in Systemic Lupus Erythematosus: an observational longitudinal study.** Arthritis Research & Therapy (submitted).

IV. Compagno M; Gullstrand B; Lood C; Sjöwall C; Jönsen A; Truedsson L; Sturfelt G; Bengtsson AA - **Osteopontin and S100A8/A9 as potential biomarkers in Systemic Lupus Erythematosus: an observational longitudinal study.** Manuscript.

Abbreviations

aCL=	anti-cardiolipin antibodies
ACR=	American College of Rheumatology
ANA=	Anti-nuclear antibody
aPL=	Anti-phospholipid
APC=	Antigen-presenting cell
APRIL=	A proliferation-inducing ligand
APS=	aPL antibodies syndrome
AUC=	Area under the curve
BAFF/BLys=	B cell activating factor/B lymphocyte stimulator
BILAG=	British Isles Lupus Assessment Group
CB-CAPs=	Cell bound complement activation products
CCP=	Cyclic citrullinated peptide
CD=	Cluster of differentiation
CDR=	Complementary determining region
CF=	Complement factor or Cystic fibrosis
CHB=	Congenital heart block
CI=	Confidence interval
CLE=	Cutaneous lupus erythematosus
CLIFT=	Crithidia Luciliae immunofluorescence test
CNV=	Copy number variation
CR=	Complement receptor
CREM=	cAMP-responsive element modulator
CRISP=	Cysteine-rich secretory proteins
cSLE=	Childhood onset SLE
CTL=	Cytotoxic T lymphocyte
DAF=	Decay accelerating factor

DC=	Dendritic cell
DNA=	Deoxyribonucleic acid
ELISA=	Enzyme linked immunosorbent assay
EM=	Electronic microscopy
FcγR=	Fragment crystallisable gamma receptor
FITC=	Fluorescein isothyocianate
FOXp3=	Forkhead box p3
G-CSF=	Granulocyte colony stimulating factor
GN=	Glomerulonephritis
GP=	Glycoprotein
HLA=	Human leukocyte antigen
HMGB1=	High mobility group box 1
IC=	Immune complex
IF=	Immunofluorescence
IFN=	Interferon
Ig=	Immunoglobulin
IP-10=	Interferon gamma-induced protein 10
IRAK=	Interleukin 1 receptor associated kinase
IRF=	IFN regulatory factor
LA=	Lupus anticoagulant
LDG=	Low density granulocytes
LE=	Lupus Erythematosus
LGL=	Large granular lymphocytes
LM=	Light microscopy
LN=	Lupus nephritis
LPS=	Lipopolysaccharide
MAC=	Membrane attack complex
MAP=	Mitogen activated protein
MASP=	Mannose associated serine protease

MBL= Mannose-binding lectin

MCP= Monocyte chemotactic protein

MHC= Major histocompatibility complex

MMP= Matrix metallo-protease

MPO= Myelo-peroxydase

MRG= Multicenter rheumatic group

MRP= Myeloid related protein

MyD88= Myeloid differentiation primary response gene 88

NCM= Necrotic cell material

NET= Neutrophil extracellular trap

NF-κB= Nuclear factor κB

NGAL= Neutrophil gelatinase associated lipocalin

NK= Natural killer

NLE= Neonatal Lupus erythematosus

NMDA= N-methyl-D-aspartate

NP= Neuro-psychiatric

NR= NMDA receptor

OPN= Osteopontin

OR= Odds ratio

PAMP= Pathogen-associated molecular pattern

PBMC= Peripheral blood mononuclear cell

PCA= Principal component analysis

pDC= Plasmacytoid DC

PI= Propidium iodide

PI3K= Phosphatidylinositol-4,5-bisphoshate 3-kinase

PMN= Polymorphonuclear neutrophil

PNC= Phagocytosis of necrotic cell

PPV= Positive predictive value

Pr/Cr= Protein-Creatinin index

PRR= Pattern recognition receptor

PTPN= Protein tyrosine phosphatase N

RAGE= Receptor for advanced glycation endproducts

RIA= Radio immuneassay

RNA= Ribonucleic acid

ROC= Receiver operating characteristic

ROCK= Rho-associated protein kinase

ROS= Reactive oxygen species

SIBLING= Small integrin-binding ligand N-linked glycoprotein

SLE= Systemic lupus erythematosus

SLEDAI= SLE disease activity index

SLICC= Systemic Lupus International Collaborating Clinics

SNPs= Single nucleotide peptides

STAT= Signal transducer and activator of transcription

STS= Serologic tests for syphilis

TACI= Transmembrane activator and cyclophilin ligand interactor

T_C= T cytotoxic

TCR= T cell receptor

T_{FH}= T follicular helper

TGF= Transforming growth factor

T_H= T helper

TIRAP= toll-interleukin 1 receptor (TIR) domain containing adaptor protein

TLR= Toll-like receptor

TRAM= TLR 4 adaptor protein

T_{Reg}= T regulator

TRIF= TIR-domain containing adapter inducing IFN-β

TWEAK= TNF-related weak inducer of apoptosis

WBC= White blood cell

Systemic Lupus Erythematosus

SLE is the internationally accepted acronym for systemic lupus erythematosus. It is a rheumatic disorder with unknown etiology, characterized by chronic or episodic inflammation in several organ systems, above all skin, joints, kidneys, nervous system and blood cells, therefore "systemic". The term "lupus" is Latin for "wolf", a word used for the first time in the 12th century by the Italian surgeon Rogerius Frugardi, or maybe even earlier by the writer Herbemius of Tours to describe the typical facial lesions in patients, reminding the marks left by a wolf´s bite[1] [2]. "Erythematosus" is Greek for "reddish", used for the first time by the French physician Cazenave to describe the color of the skin rash[1].

SLE is considered as a prototypical autoimmune disease, which means that the immune system attacks the body´s own tissues. The impairment of different parts of the immune system is crucial to the pathogenesis of SLE. The role played by the adaptive immunity was addressed already in the 1950´s, when the presence of different autoantibodies in biologic samples became the hallmark of SLE[3] [4] [5]. Concerning the innate immunity, besides the complement system[6-10], different cells are also involved in the complex pathogenesis of SLE, such as neutrophils, monocytes and dendritic cells (DC) and their functions have been elucidated more recently[11-18]. The impaired mechanisms of cell death have been shown to provide a crucial contribution to the production of autoantigens, holding on a vicious circle and leading to recurrent clinical manifestations[19-23].

Epidemiology

To report the exact frequency and distribution of SLE in the population is challenging. It depends upon the different ways to identify and ascertain the diagnosis in the different epidemiological studies available in literature.

SLE affects more than 5 million people all over the world. The prevalence rates have varied between 3 and 207 cases per 100,000 inhabitants and the incidence rates from 2 to 6 new cases per 100,000 per year, with variation by sex, race, ethnicity and socioeconomic status. Generally, rates are higher in women than

men and in African Americans as compared to Caucasians[24] and the prevalence is high among Asians, Hispanics and Native Americans[25].

According to national register data, in 2010, the overall prevalence of SLE in Sweden varied by county ranging from 46 to 85 cases per 100,000 inhabitants, depending on the stringency of diagnostic method, with range 79-144 among females and range 12-25 among males. The prevalence is lower (2 per 100,000) in children (age group 0-14 years) and increases to 52 in age group 15-49 years, and up to 95 per 100,000 in people >/=50 years[26]. Previous data from southern Sweden reported a prevalence of 68/100,000 inhabitants and an incidence of 4.8/100,000 inhabitants per year[27 28].

Up to 90% of patients affected by SLE are women, who often are affected in childbearing age. Patients with SLE have greater weekly absenteeism than controls with similar jobs, with higher work disability in African American SLE patients, especially the older ones and with less formal education[29].

Increased mortality rate as compared to the general population has also been documented in patients with SLE. Common causes of death recorded in SLE patients are malignancies, severe infections, thrombosis and cardiovascular diseases, besides active disease[30-33]. How the use of immunosuppressive treatments affects the mortality rate is controversial. The introduction of effective pharmacological treatment has dramatically prolonged the survival of SLE patients, but it has also increased the risk of severe infections and perhaps the risk of malignancies and cardiovascular diseases[33-35].

Etiology

The etiology of SLE is not known, but it is clear that genetic and environmental factors contribute to the development of the disease. Autoimmune diseases are generally more common among women as compared to men, which may depend on hormones, in particular sex hormones as far as SLE is concerned.

Genetics

Development of SLE is seen in patients with genetic deficit of the complement components of the classical pathway (C1q, C2, or C4) [9 36 37]. About 75% of patients with the rare complete C4 deficiency develop a lupus-like condition[38], probably because of defect in clearance of apoptotic material, generating autoantigens and production of pathogenic autoantibodies. Studies in families with multiple members affected by SLE and genome-wide association studies (GWAS)

have contributed to the detection of over 50 different loci associated with SLE susceptibility[39]. Monozygotic and dizygotic twins have 25% and 2% concordance for SLE, respectively[40 41]. Strongest genetic associations were found with IRF5[42], MHC, PTPN22[43], FCγRIIA, and STAT4[44-46]. HLA-DR3, DR9 and DR15 have been associated with lupus nephritis[47].

Men with Klinefelter's syndrome have a more than tenfold higher risk of developing SLE than other men, whereas females with Turner's syndrome (XO) are protected from the disease[48].

Environment

Exposition to sunlight is not recommended to SLE patients because of increased risk of flare, but the pathogenic role of ultraviolet radiation in susceptible healthy individuals is controversial[49].

The role played by infections, especially caused by Epstein-Barr virus (EBV) and cytomegalovirus (CMV), in the etiology of SLE and in triggering flares has been extensively debated[50-52]. DNA and RNA from viruses are essential to stimulate the production of type I IFN, a key cytokine to SLE disease severity.

Some anti-epileptics and TNF-α blockers are known to induce the so-called "drugs-related SLE", presenting often with joint and mucocutaneous symptoms and the presence of autoantibodies, such as ANA and anti-histone antibodies[53].

Cigarette smoking confers a short-term increased risk of SLE in genetically susceptible individuals[54]. Alcohol consumption in moderate doses may have a protective effect against the development of SLE, although this is still debated[55]. Occupational exposure to unknown doses of silica, probably in combination with unknown susceptibility factors, is a well-established risk factor for SLE[56]. Controversial finding concerns pesticide and solvents[57]. In experimental models, the onset and severity of lupus-like disease are not altered when comparing germfree and conventionally raised mice[58].

Hormones

The disease is much more prevalent in women than men, especially in the fertile age. The risk of flares after use of sex hormones as contraceptives or hormone-replacement therapy (HRT), as well as in physiological conditions such as pregnancy and puerperium is increased. Hypoandrogenism has been described in men with SLE, and androgen therapy is sometimes recommended for the treatment of some SLE manifestations[59].

Clinical features

SLE should be considered as a syndrome rather than as a disease, as it may present different phenotypes and it may affect several organ systems during its course. The lack of exclusive manifestations makes the formulation of SLE diagnosis difficult, especially in an early phase of the disease.

The most relevant clinical manifestations observed in SLE patients are shortly discussed in the next paragraphs. They are included in the international classification criteria that have been available since 1971[60] and periodically updated later[61-63]. All the different classification criteria validated over the years are summarized in table 1. They should be used for classification of patients included in scientific studies, but they are often used in the diagnostic process in clinical practice. Official international diagnostic criteria for SLE are still missing[64].

It is common that SLE patients develop a combination of chronic inflammation in different organ systems with specific immunological abnormalities, during the natural course of the disease and oligo-symptomatic patients, not fulfilling classification criteria for the disease, could still have the clinical diagnosis SLE.

The course of the disease is typically irregular, with "calmer" periods of low disease activity or clinical remission, alternating with "flares", when the inflammation in target tissues is triggered and the activity of the disease increases. A way to follow the activity of the disease is to make use of tailored tools, such as the SLE disease activity index 2000 (SLEDAI-2K)[65] or the BILAG (British Isles Lupus Assessment Group) 2004 index[66], where the presence or absence of each clinical manifestation contribute to the calculation of a score, mirroring the disease activity. The higher is the score, the more active is the disease. SLEDAI-2K is widely used and validated, but it does not record improving or worsening and it does not include severity within an organ system. Other scores are sometimes used for the assessment of disease activity in SLE patients, such as ECLAM (European Consensus Lupus Activity Measurements), SLAM-R (Systemic Lupus Activity Measure, Revised), SLAQ (Systemic Lupus Activity Questionnaire for Population Studies) and SDI (Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index)[67].

Table 1. SLE classification criteria over the years.

ACR 1971	ACR 1982	ACR 1997	SLICC 2012	
			Clinical criteria	Immunologic criteria
Malar rash	Malar rash	Malar rash	Acute or subacute CLE	ANA > lab reference range
Discoid rash	Discoid rash	Discoid rash	Chronic CLE	Anti-dsDNA
Photosensitivity	Photosensitivity	Photosensitivity	Non scarring alopecia	Anti-Sm
Oral ulceration	Oral ulceration	Oral ulcers	Oral/nasal ulcers	aCL, anti-β2GP1, LA or false + STS
Arthralgia, arthritis	Arthritis	Arthritis	Arthritis	Low C3, C4 or CH50
Pleuritis, pericarditis	Pleuritis, pericarditis	Pleuritis, pericarditis	Pleuritis, pericarditis	Direct Coomb´s test +
Proteinuria >3.5 g/d	Proteinuria >0.5 g/d or cellular casts	Proteinuria >0.5 g/d or cellular casts	Urine Pr/Cr or 24 h proteinuria >500 mg/d or red blood cell casts	
Cellular casts	Psychosis, seizures	Psychosis, seizures	Psychosis, seizures, mono-neuritis multiplex, myelitis, peripheral/cranial neuropathy, cerebritis	
Psychosis, seizures	Hemolytic anemia, leukopenia, lymphopenia, thrombocytopenia	Hemolytic anemia, leukopenia, lymphopenia, thrombocytopenia	Hemolytic anemia	
Hemolytic anemia, leukopenia, thrombocytopenia	LE cells, false + STS, anti-dsDNA, anti-Sm	aCL or LA, False + STS, anti-dsDNA, anti-Sm	Leukopenia, lymphopenia	
LE cells	Antinuclear antibody	Antinuclear antibody	Thrombocytopenia	
False + STS				
Raynaud´s phenomenon				
Alopecia				

STS= Serologic test for syphilis
GP= Glycoprotein
LE= Lupus Erythematosus
LA= Lupus anticoagulant
CH= Total complement activity
ds= Double stranded
CLE= Cutaneous Lupus Erythematosus
aCL= Anti-cardiolipin
Pr/Cr= Protein/Creatinin index

Mucocutaneous manifestations

The skin manifestations occur in about 70% of SLE patients[68] and may be present without systemic involvement, the clinical entity referred to as cutaneous lupus erythematosus (CLE). CLE is slightly more common than SLE in women, but much more common than SLE in men[69]. Lupus erythematosus was considered as a cutaneous disease up until 1872, when Kaposi described the occurrence of constitutional symptoms in some patients[70]. Malar rash and discoid rash are the most typical skin lesions, but several different, less specific manifestations often affect SLE patients. The most frequent mucocutaneous manifestations in SLE are summarized in table 2.

Table 2. Most frequent mucocutaneous manifestations in SLE patients.

Malar rash or butterfly rash or localized acute cutaneous lupus erythematosus (ACLE)	Oral or nasal ulcers
Generalized ACLE	Urticaria
Subacute cutaneous lupus erythematosus (SCLE)	Alopecia (frontal or diffuse non-scarring)
Discoid rash or chronic cutaneous lupus erythematosus (CCLE or DLE)	Vascular lesions (Raynaud´s phenomenon, livedo reticularis, dermal vasculitis, etc)
Photosensitivity	Sclerodactyly

Musculoskeletal manifestations

Arthropathy, often symmetric and resembling the one occurring in other rheumatic diseases, such as Rheumatoid Arthritis (RA), is reported in 76-98% of SLE patients[71-74] and may affect both small and large joints, with or without evident signs of local inflammation. The most characteristic articular manifestation of SLE is a non-deforming, non-erosive arthritis. A minority of patients can be affected by correctable deformities in hands and wrists, the so-called "Jaccoud´s arthropathy". Erosive arthritis has seldom been reported in SLE patients, often in association with overlapping disorders and presence of anti-CCP antibodies[75]. Other musculoskeletal manifestations include myalgia, myositis and osteonecrosis (most commonly of the femoral head).

Cardiac and pulmonary manifestations

SLE can affect all the layers (pericardium, myocardium and endocardium) of the heart, but cardiac involvement determines relatively uncommon clinical features. At autopsy nonetheless, in up to 80-100% of SLE patients, heart lesions can be found. Pericarditis is one of the most characteristic disease manifestations and echocardiography based studies show pericardial abnormalities in up to 54% of

SLE patients, mostly at disease onset or during SLE relapses[76]. Cardiac tamponade, constrictive and purulent pericarditis are rare. Libman-Sacks endocarditis is the most characteristic valvular lesion, but valvular thickening and regurgitation are more frequent[76]. Myocarditis is reported in up to 10% of SLE patients, where the myocardial dysfunction may also be the consequence of coronary artery disease (CAD), mostly due to premature atherosclerosis. CAD occurs in up to 10% of SLE patients, with higher risk of angina pectoris, myocardial infarction and sudden death[76]. Sinus tachycardia is a very frequent rhythm abnormality in SLE, whereas conduction disturbances, such as atrio-ventricular block and bundle branch block, are seldom observed[76].

Pulmonary manifestations may be the presenting symptoms in 4-5% of SLE patients. The involvement of any component of the respiratory system (airways, vessels, parenchyma, pleura and respiratory muscles) affects around half of patients during the disease course[77]. Pleuritis and pulmonary infections are the most prevalent manifestations. Infrequent manifestations include interstitial lung disease, acute lupus pneumonitis, diffuse alveolar hemorrhage, pulmonary arterial hypertension and shrinking lung syndrome[78].

Renal manifestations

Several different types of renal manifestations may be found in patients affected by SLE, often with deposits of immune complexes or complement factors in glomeruli, tubuli, interstitium and renal vessels, which characterizes the clinical phenotype often referred to as lupus nephritis (LN). The clinical presentation of LN may vary from mild abnormalities of the urine analysis to manifest nephrotic or nephritic syndrome. Periods of clinical remission alternate with unpredictable flares.

The gold standard to diagnose LN is the histology analysis after renal biopsy. The most typical histological changes detectable in LN were classified in 1974, 1982 and 1995 by the World Health Organization (WHO) and later revised in 2003 by the International Society of Nephrology (ISN) and the Renal Pathology Society (RPS)[79]. The main features of the different international classifications of renal changes are summarized in table 3.

Table 3. Classifications over years of renal changes in patients affected by lupus nephritis

Class	1974	1982	1995	2003
I	Normal glomeruli	Normal glomeruli a) By all techniques b) Only by LM, but deposits by EM or IF	Normal glomeruli a) By all techniques b) Only by LM, but deposits by EM or IF	Minimal mesangial nephritis Normal glomeruli by LM, but mesangial immune deposits by IF
II	Purely mesangial disease a) Normal mesangium by LM b) Mesangial hyper-cellularity	Pure mesangial alterations a) Mild hyper-cellularity b) Moderate hyper-cellularity	Pure mesangial alterations a) Mesangial widening and/or mild hyper-cellularity b) Mesangial cell proliferation	Mesangial proliferative nephritis
III	Focal proliferative GN (<50%)	Focal segmental GN a) "Active" necrotizing lesions b) "Active" and sclerosing lesions c) Sclerosing lesions	Focal segmental mesangio-capillary proliferative GN (<50% glomeruli) a) Active necrotizing lesions b) Active and sclerosing lesions c) Sclerosing lesions	Focal nephritis (A): active proliferative (A/C): active and chronic proliferative and sclerosing (C): chronic inactive sclerosing with glomerular scars
IV	Diffuse proliferative GN (≥50%)	Diffuse GN a) Without segmental lesions b) "Active" necrotizing lesions c) "Active" and sclerosing lesions d) Sclerosing lesions	Diffuse proliferative a) Segmental lesions b) Active necrotizing lesions c) Segmental and active lesions d) Sclerosing lesions	Diffuse nephritis S (A): active segmental proliferative G (A): active global proliferative S (A/C): active and chronic segmental proliferative and sclerosing G (A/C): active and chronic global proliferative and sclerosing S (C): Chronic inactive segmental sclerosing with scars G (C): Chronic inactive global sclerosing with scars
V	Membranous GN	Diffuse membranous GN a) Pure b) Associated with category II (a or b) c) Associated with category III (a–c) d) Associated with category IV (a–d)	Diffuse membranous GN a) Pure b) Associated with category II (a or b)	Membranous nephritis with or without mesangial alterations
IV		Advanced sclerosing GN	Advanced sclerosing GN	Advanced sclerotic nephritis

LM= Light microscopy
IF= Immunofluorescence
(A)= Active
G = Global
EM= Electron microscopy
GN= Glomerulonephritis
(C)= Chronic
S= Segmental

Neurological and psychiatric manifestations

According to the American College of Rheumatology (ACR) standardized nomenclature, 19 different neuropsychiatric (NP) manifestations of SLE are grouped in 3 different categories[80]: psychiatric syndromes (including anxiety, mood disorder and psychosis); neurologic syndromes of the central nervous system (CNS); neurologic syndromes of the peripheral nervous system. The prevalence of NP manifestations in SLE patients ranges widely from 19% to 91% in the different series reported[81-84].

Headache and **mood disorders** are the most prevalent ones and have been attributed to both lupus and non-lupus causes[85 86]. The characteristics of headache and mood disorders in SLE are similar to those in the general population and have the same heterogeneity in clinical presentation. They may occur in association with other NP events[85 86].

Prevalence estimates of **seizures** in SLE have varied between 6% and 51%[81 83 87-90]. The reported range of frequency is 4.6%-6.7% in different studies[84 91 92]. Seizures often occur within 1 year after SLE diagnosis and SLE patients who have seizures use to have more active and more severe disease course[84].

Psychosis is not a common feature in SLE, with a prevalence varying from 0% to 11%[81-84 93]. Psychosis is usually an early finding in the course of the disease or occurs within the context of florid activity of the disease, associated often with cutaneous and hematological manifestations. Psychiatric symptoms can precede the onset of lupus and are rarely a late complication of the disease[93].

The **less common neuropsychiatric manifestations** of SLE are aseptic meningitis, cerebrovascular disease, demyelinating syndrome, chorea, myelopathy, acute confusional state, cognitive dysfunction, Guillain-Barre´s syndrome, mononeuritis multiplex, autonomic disorder, myasthenia gravis, cranial neuropathy, plexopathy and polyneuropathy[80].

Hematological manifestations

A review of the main hematological manifestations in SLE patients has recently been published[94] and is summarized in this paragraph.

Anemia is a common hematological feature in SLE, more often the anemia of chronic disease, normocytic and normochromic, resulting from suppressed erythropoiesis secondary to chronic inflammation. Autoimmune hemolytic anemia may affect up to 10% of SLE patients, often associated with other severe SLE-related manifestations. It is characterized by elevated reticulocyte counts, low

haptoglobin levels, increased indirect bilirubin concentration and a positive direct Coombs' test. Another common type is the iron deficiency anemia, whereas pure red cell aplasia, pernicious anemia and aplastic anemia have rarely been reported in SLE patients.

Leukopenia can be due to lymphopenia, neutropenia or a combination of both. The prevalence of lymphopenia in SLE ranges from 20 to 81% and its degree may correlate with disease activity. Neutropenia is a common feature of SLE, with a prevalence rate of 47%. Immunosuppressive agents like Azathioprine or Cyclophosphamide have the potential to worsen leukopenia via bone marrow suppression.

Thrombocytopenia has a reported prevalence ranging from 7 to 30% and it may be acute in onset and extremely severe, but the chronic form is more common. Increased peripheral destruction of platelets and the presence of anti-platelet antibodies (anti-phospholipids or other antibodies in some patients) are the most likely pathogenic mechanisms. Thrombocytopenia is an independent risk factor for increased mortality in SLE. Idiopathic thrombocytopenic purpura (ITP) may predate by up to 10 years the onset of SLE in up to 16% of patients. Thrombotic thrombocytopenic purpura (TTP) is a rare but life-threatening complication in SLE, characterized by hyaline thrombi in many organs, neurologic abnormalities, renal insufficiency, and fever combined with thrombocytopenia. Detection of the fragmented peripheral red blood cells helps in early diagnosis of TTP.

Pancytopenia may result from bone marrow failure, such as in the case of aplastic anemia. Macrophage activation syndrome, although unusual has been reported in SLE. Enlargement of lymph nodes occurs in approximately 50% of patients with SLE. It is more frequently noted at disease onset or during exacerbations. Splenomegaly occurs in 10%-46% of patients, particularly during active disease.

Association with other diseases

Many diseases, autoimmune or not, rheumatic or not, are relatively common in patients affected by SLE, being some of them crucial in prognostic terms. Frequent co-morbidities are represented by osteoporosis and vascular damage. The latter occurred in 26% of SLE patients in a Caucasian cohort with follow-up of almost 12 years[95].

Accelerated **atherosclerosis** and its long-term sequelae represent major causes of late mortality among patients with SLE. Aggressive management of all traditional Framingham risk factors (hypertension, hypercholesterolemia, diabetes, and smoking) is recommended. Statins seem to have no effect on cardiovascular outcomes in adult or pediatric SLE populations[96].

Anti-phospholipid (aPL) antibodies are not associated with atherosclerosis, but are present in about one third of SLE patients. They can determine, instead, the **aPL antibodies syndrome** (APS), characterized by thrombocytopenia, recurrent abortion, arterial and venous thrombosis. APS affects up to one third of SLE patients. In one of the latest reports from a large cohort of patients, anti-cardiolipin antibodies were found in 47%, anti-β2-glycoprotein I in 32.5% and lupus anticoagulant (LA) in 26%. Patients with LA at baseline have 50% odds of a deep venous thrombosis/pulmonary embolus in the next 20 years. LA is the only aPL antibody strongly associated with myocardial infarction[97].

Osteoporosis is a common complication of SLE. The prevalence of fractures in relatively young SLE patients is high, but it might be explained by the interplay between the systemic inflammation with SLE-related complications, such as renal bone disease, or its treatment, above all glucocorticoids[98].

Fibromyalgia is found in around one fourth of SLE patients, a prevalence that is slightly higher than in patients with arthritis[99]. Fibromyalgia shares many symptoms with SLE and is the source of much of the disability. Fibromyalgia does not correlate with SLE disease activity, but the clinical features in these patients may contribute to a misinterpretation of lupus activity[100].

A somewhat increased incidence and overall risk of **malignancy** with a reduction in survival was recently reported, being prostate cancer and cervical cancer more prevalent in SLE patients. Moreover, a prominent (3-fold) increased risk of non-Hodgkin lymphoma is documented, whereas breast cancer is less frequent than in the general population. Inadequate viral clearance in SLE could promote the development of certain malignancies such as cervical cancer. It is controversial whether the inherent SLE activity is a risk factor for cancer. No drug dose or duration of treatment was identified to be involved in the increased risk of malignancy in SLE[101]. Cancer preventive methods are important in the SLE population[102].

Childhood onset SLE

About 15-20% of cases of SLE are diagnosed in patients younger than 16 years, which characterizes the childhood onset SLE (cSLE). The usual age of onset is between 12 and 14 years of age and age below 5 years is very rare.

In a couple of reviews comparing cSLE to adult onset SLE, it is referred that cSLE is characterized by a more frequent acute or fulminant onset, an increased male-to-female ratio (about 20% males), a higher prevalence of nephritis, hematological manifestations and CNS involvement, a higher prevalence of progression to end-stage renal disease (ESRD), lower prevalence of pulmonary involvement, arthritis

and discoid lupus compared to adult-onset SLE patients. Pediatric patients may experience a serious negative impact on their psychosocial and physical development, growth delay, osteoporosis, the psychological effect of steroid-induced alterations of the physical image, and often poor treatment compliance[103] [104].

Neonatal SLE

Neonatal lupus erythematosus (NLE) is a rare acquired autoimmune disease caused by trans-placental transfer of maternal IgG autoantibodies to the fetus, regardless of the mother´s health status. The fetuses are identified with congenital heart block (CHB) in a structurally normal heart. The majority of cases are associated with maternal Ro/SSA and La/SSB antibodies. The risk for a woman with presence of the candidate antibodies to give birth to a child with CHB is estimated around 1 in 50. While the precise pathogenic mechanism of antibody-mediated injury remains unknown, it is clear that the antibodies alone are insufficient to cause disease and fetal factors are likely contributory, such as apoptosis of cardiocytes with surface translocation of Ro and La antigens. The immune complexes formed after binding of maternal autoantibodies stimulate macrophages to secrete pro-fibrosing factors, such as TGF-β. Varying degrees of heart block, with both early and late onset have been described, as well as the post-natal progression of incomplete blocks, despite the clearance of the maternal antibodies from the neonatal circulation[105]. Neonatal lupus due to anti-ribonucleoprotein (RNP) antibodies has rarely been reported, with varicelliform lesions at birth and without cardiac involvement[106].

Treatment

The involvement of vital organs was a common cause of mortality in SLE patients, before the introduction of effective treatments. Across the last decades, several different therapeutic approaches have been proposed for the treatment of SLE. The prognosis has improved a lot since then, but the disease at present is still incurable.

Being a multifaceted systemic disease, the therapeutic protocols in SLE may vary in relation to the clinical phenotype in the individual patient.

The inflammatory component characterizing the acute phases of the disease is often treated with adequate doses of **corticosteroids** that modulate many of the immune pathways through inhibition of NF-κB[107].

The vast majority of the SLE patients is recommended to use low-dose corticosteroids along with a maintenance dose of **anti-malarials** as lifelong treatment. One possible mechanism of action of anti-malarials is through the inhibition of TLR-signalling and the downstream production of IFNα[108].

More aggressive clinical course, with involvement of vital organs and recurrent increase of disease activity are standard indications for treatment with **immunosuppressive drugs**, such as Azathioprine[109], Cyclosporine A[110], Cyclophosphamide[111], Methotrexate[112], Mycophenolate Mofetil[109] and Tacrolimus[110]. The immunosuppressive drugs inhibit DNA synthesis in diverse ways and thus prevent expansion of activated immune cells. They have improved the outcome in SLE patients, indeed, but the suppression affects often the whole immune system and the adverse events, first of all infections, are sometimes severe and life-threatening.

Since the beginning of the third millennium, the search for new drugs has been focusing on the development of treatments that more specifically target, modulate or block key parts of the imbalanced immune system, the so-called "**biologic treatment**".

The most used biologic drugs in the treatment of SLE patients target with monoclonal antibodies the B-cells, either blocking the cell surface receptor CD20 (Rituximab)[113], or antagonizing the soluble mediator BLyS or BAFF (Belimumab)[114]. Epratuzumab (anti-CD22 antibodies)[115] is the newest biologic treatment that targets B-cells, with evidence of efficacy and tolerability in SLE patients, but its routine clinical use has not started, yet.

The evidence regarding the other biologics, such as those targeting IFN or IL-6, is currently limited[116]. The use of TNF-α antagonists, such as Infliximab[117] and

Etanercept[118], is more restricted to the clinical phenotypes characterized by dominant musculoskeletal involvement, but it is still controversial in other patients, since these molecules are also included in the long list of potential triggers of drug-induced SLE[116]. In clinical trials, the T cell co-stimulation modulator Abatacept was well tolerated and showed evidence of biologic activity compared to placebo, but the achieved clinical improvement did not meet the primary endpoints of the investigations[119 120].

Hematopoietic and mesenchymal stem cell transplantation for severe and refractory systemic lupus erythematosus[121 122], as well as therapies targeting new pathogenic pathways will perhaps be included in the SLE treatment in the next decades.

Prognosis

The introduction of effective treatments has changed the prognosis of patients affected by SLE. The better knowledge of the disease and of its complications has also contributed to tailor the management of the patients with positive results in term of reduced co-morbidity, improved survival and better quality of life.

Before the introduction of corticosteroids, the 5-year survival was less than 50%, in comparison to current 10-year survival around 90%[123]. A recent contribution from the largest lupus cohort reports the overall cumulative probability of survival after disease diagnosis at 5, 10, 15, and 20 years to be 95%, 91%, 85%, and 78%, respectively[124].

Reports in the past have suggested male gender, poor socioeconomic status, juvenile onset of disease, African-American ethnicity, presence of lupus nephritis or other relevant co-morbidity as risk factors for worse prognosis. More recently, the severity of late-onset disease has been emphasized as well as the irrelevant difference between males and females in the long-term outcome[125]. Among the clinical manifestations and the laboratory variables, only hemolytic anemia and hypocomplementemia seem to be associated with poor prognosis[124].

The main causes of death are cardiovascular events[27], especially in men, and infections, especially in women[125]. The disease related complications are rarely mentioned as causes of death in the current literature. Nonetheless, in a recent article[126], the quality of the majority of prognosis studies in SLE patients was criticized, because of the lack of rigorous study design, especially in addressing confounding factors, study participation and attrition, as well as inadequate handling of missing data.

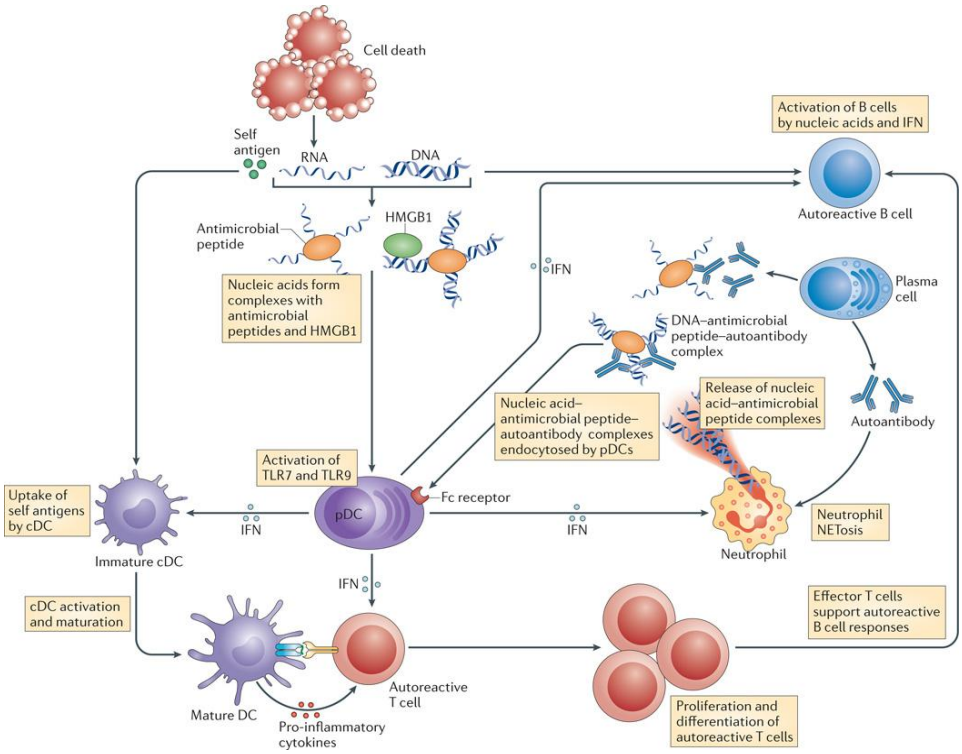
Pathogenesis

Many aspects of SLE pathogenesis are nowadays known, but there are still many questions left about what lies beneath the acute and chronic phases of inflammation, the flares alternating with remission periods and the mechanisms that the physician try to inhibit or block with the help of the available therapeutic armamentarium.

Very briefly, one could say that genetic, hormonal and environmental factors determine, in SLE patients, a chronic impairment of the immune system, mainly characterized by the complex interrelation of the following factors: cell death dysfunction, abnormal production of cytokines and T-cells and B-cells dysfunction. Which disorder comes first is not known, but the end result of the mentioned combination is the production of a multitude of autoantibodies, the activation of the complement system and the inflammatory response. A plausible explanation of SLE pathogenesis is that the patients have an increased rate of cell death and a dysfunctional disposal of the cell waste materials, which drive the formation of autoantigens and consequent hyperactive production of autoantibodies. The resulting formation of circulating immune complexes leads to activation and consumption of complement components, which propagate the vicious circle, by further reducing the capability of clearing apoptotic cells.

Moreover, some cells of the innate immunity, namely neutrophils, monocytes/macrophages and dendritic cells, play important pathogenic roles in SLE. Marked abnormalities in phenotype and function affect polymorphonuclear (PMN) neutrophils in SLE patients. Upon PMN activation, pro-inflammatory molecules, among others S100A8/A9, are released. Neutrophils death through apoptosis and NETosis is enhanced in SLE patients and the release of nuclear material might also be a potent source of autoantigens. A distinct subset of pro-inflammatory low-density granulocytes (LDG), isolated from patients with SLE, induces vascular damage, displays enhanced bactericidal gene signatures and synthesizes increased amounts of type I IFNs[127].

The engulfment of the immune complexes by plasmacytoid dendritic cells (pDC), leads to production of type I interferons (IFN), which can stimulate B cells to further production of autoantibodies[128 129].



Dying cells release nucleic acids; these form large complexes with antimicrobial peptides (such as LL37) and with endogenous DNA-binding proteins (such as high mobility group protein B1, or HMGB1). These DNA and RNA complexes activate pDCs via TLR9 and TLR7, respectively, and they induce the production of type I IFN. In turn, type I IFN promotes T cell activation, autoantibody production by B cells and the release of neutrophil extracellular traps (NETs). Autoreactive antibodies activate neutrophils and form DNA-containing immune complexes that are preferentially endocytosed by pDCs via Fc receptors. Self-nucleic acids also activate classical DCs (cDCs) and they promote the release of inflammatory cytokines and the priming of T cells that are specific for self antigens in a process that is also facilitated by type I IFN.

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Apoptosis and NETosis

Many cells die and are removed and replaced every day, most often through a highly regulated process called apoptosis, even known as programmed cell death. The dying cell exposes lots of 'eat me' molecules (such as phosphatidylserine) and excretes 'find me' signals. The scavenger cells are thus recruited to the dying cell and remove it, with the help of complement components, which induces an anti-inflammatory response.

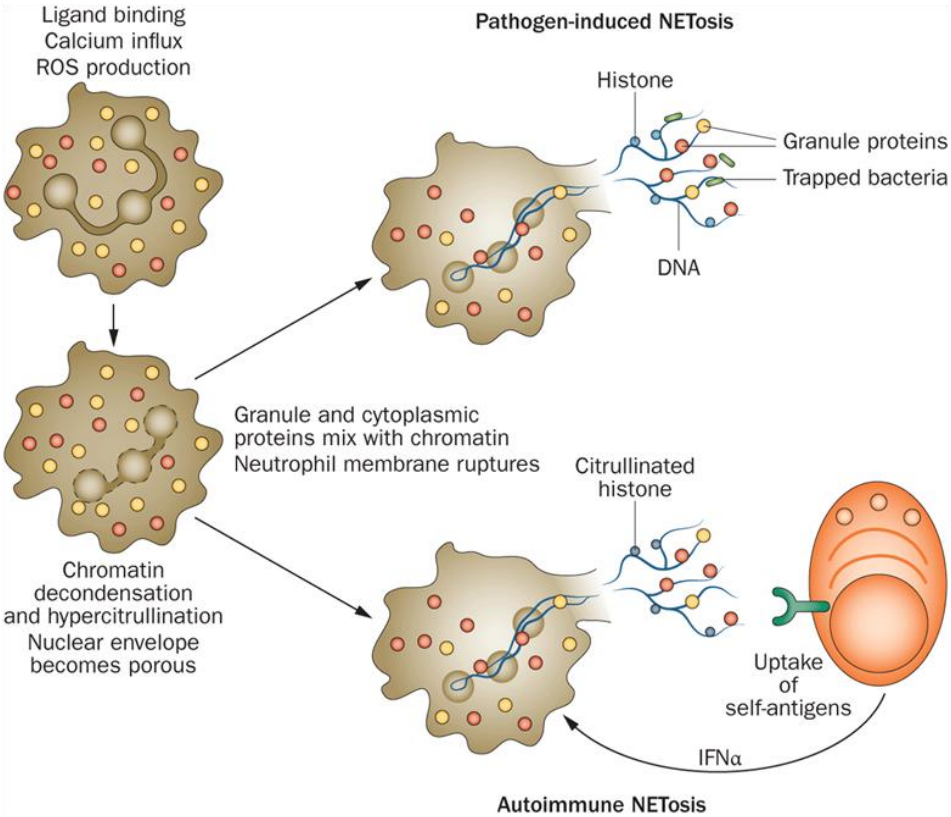
The neutrophils die also by apoptosis in physiological conditions, but upon activation they can die by a process referred to as NETosis, after releasing extracellular traps (NET). NETs are composed of nuclear components, such as DNA and histones, associated with granular proteins. The production of reactive oxygen species (ROS), the histone citrullination and the translocation of some of the granular proteins seem to be central events leading to NET formation[130]. After stimulation of receptors, neutrophils adhere to the substrate and mobilize granule components, histones in the nucleus get processed, and the intracellular membranes disintegrate. Finally, the cell membrane ruptures, and the mixture of cytoplasm and nucleoplasm gets expelled to form NETs[13]. NETs immobilize pathogens, thus preventing them from spreading, but also facilitating subsequent phagocytosis of trapped microorganisms. The antimicrobial histones and proteases in NETs can also directly kill pathogens.

Apoptosis and NETosis in SLE

Increased amount of dying cells and reduced capability to remove them, because of complement consumption and dysfunctional macrophages, are probably involved in the pathogenesis of SLE. Some apoptotic cells progress thus into secondary necrosis, with loss of plasma membrane integrity and exposition of intracellular and nuclear antigens. Likewise, it has been suggested that NETosis is increased and the degradation and removal of NETs are reduced[131]. In addition, in SLE patients, a distinct subset of neutrophils, the so-called low-density granulocytes[17], with intrinsic increased capability of releasing NETs, has been described. All above may contribute to initiating the break of self-tolerance and lead to formation of autoantigens.

The engulfment of immune complexes and NETs from SLE patients can activate plasmacytoid dendritic cells (pDCs), in the presence of the anti-microbial peptides LL-37 and HMGB1[11], leading to an increased secretion of pro-inflammatory cytokines, such as interferon (IFN)-α, IFN-γ-induced protein 10 (IP-10), TNF-α

and IL-6[132]. The presence of autoantibodies induces production of type I IFNs and the formation of large immune complexes and aggregates that can be trapped in the tissues and activate the complement system, cause leukocyte infiltration, inflammation and tissue destruction.



Release of NETs can be considered a specialized form of cell death, termed NETosis. The process is induced by ligand binding, and involves calcium influx and ROS production. Chromatin becomes hypercitrullinated, leading to its decondensation. Pores form in the nuclear membrane and secretory vesicle walls, enabling granule and cytoplasmic proteins to mix with the chromatin. Finally, the neutrophil cell membrane ruptures, extruding the NET, which is composed of nuclear material (DNA and histones) as well as granule enzymes (myeloperoxidase, elastase, lactoferrin, MMP-9) and cytoplasmic proteins (LL37). These proteins serve as antimicrobial agents during pathogen-induced NETosis, but may be a source of autoantigens during autoimmune NETosis, in diseases such as systemic lupus erythematosus and rheumatoid arthritis. Abbreviations: MMP, matrix metalloproteinase; NET, neutrophil extracellular trap; ROS, reactive oxygen species.

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Innate and adaptive immune systems

The human immune system has the crucial role to protect us from pathogens and toxins in the environment. It is challenging to summarize the multitude of components and mechanisms involved in the immune system that interplay in a very fascinating and complex manner. A way to make a very long story much shorter is to try to divide the many components of the immune system into two main functional groups, the so-called “innate immunity” and “adaptive immunity”. Within each of these two groups, it is possible to distinguish a “humoral” from a “cell-mediated” component.

The main differences between the innate and the adaptive immunity are related to the response to stimulation from pathogens, antigens and toxins. The innate immunity is always active and provides the system with the same immediate, but unspecific, limited and relatively weak response against pathogens. The adaptive immunity is normally silent, reacts slowly against pathogens, but with highly specific and potent response.

The role of the innate immunity is to immediately mobilize and fight pathogens at the site of infection. The main actors in the humoral innate part of the immune system are the complement system and the secreted pattern recognition receptors (PRR), whereas the cellular components are neutrophils, monocytes, macrophages, natural killers (NK) and dendritic cells (DC).

The adaptive, or specific or acquired, immune system is the part of the human immunity responsible of memorizing specific pathogens (called antigens) the first time they are encountered, in order to recognize them in the future, prevent their growth and eliminate them efficiently. It is the basis of vaccination, and it is supposed to distinguish between own (or self) and unwanted (or non-self) antigens. The impairment of adaptive immunity may result in immunodeficiency, allergy and autoimmunity.

Likewise the innate one, the adaptive immunity consists of humoral components, called antibodies or immunoglobulins, and cellular components, called lymphocytes.

Complement system

The complement system represents the most important humoral component of the innate immune system and has many immunological functions both in the protection against pathogens but also in the clearance of dying cells. In 1895, Bordet described it as a heat-labile serum component able to complement the antibacterial effect of antibodies. Since than three main activation pathways (the classical, the alternative and the lectin) of the complement system have been described, leading to the activation of a terminal pathway, with the involvement of more than 30 soluble and membrane bound proteins, several regulatory proteins and receptors.

Complement factors

Nine main components (C1-C9) are involved in the different pathways and their nomenclature is based on the order in which they were identified. C1, C2 and C4 are the main players of the classical and lectin activation pathways. C3 is the only component involved in the alternative activation pathway. C5 is the first component of the common terminal pathway, which leads to the formation of a membrane attack complex (MAC) where C6, C7, C8 and C9 are involved. The MAC determines cell lysis and destruction of the target.

Upon their activation, C3, C4 and C5 are cleaved into one smaller (called “a”) and one larger (called “b”) fragment. C2 is also cleaved but the nomenclature of its fragments is the opposite, so “a” is the larger one and “b” is the smaller one.

Other ways for the complement system to protect against pathogens are chemotaxis and opsonization. The smaller split products C3a, C4a and C5a are released into the circulation as anaphylatoxins, for recruitment of immune cells to the infected area to clear the pathogen. The larger fragments, such as C3b and C4b can opsonize pathogens and dying cells to facilitate their recognition and clearance by immune cells.

C1 is the first component of the classical pathway, consisting of a complex of five molecules, one C1q, two C1r and two C1s. C1q is the pattern recognition molecule of the C1 complex which is released mainly by macrophages and dendritic cells. It is a calcium-dependent protein, which consists of six identical subunits with globular heads and long heterotrimeric (A, B and C chains) collagen-like tails. The heads can bind to the constant regions of immunoglobulin molecules, to pentraxins such as C-reactive protein (CRP), or directly to different structures on the surface of pathogens or dying cells. The tails of C1q are bound to the heterotetramer (C1r:

C1s) 2, composed by two C1r and two C1s molecules, serine proteases responsible for the initiation of the classical pathway activation of the complement system.

Activation of the complement system

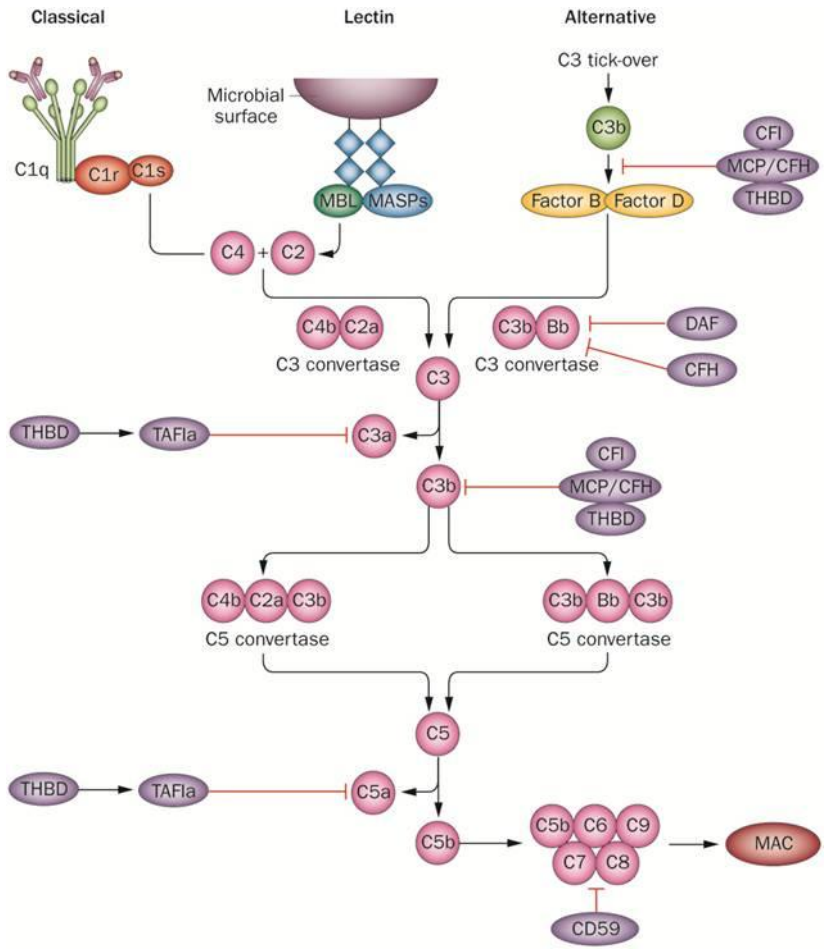
The main activators of the classical pathway are immune complexes (IC). The Fc-region of one IgM or at least two IgG (except for IgG4) molecules is recognized and bound by C1q, which determines a conformational change in the (C1r: C1s) 2 complex and activation of its components. C1s can start the cleavage of C4 and C2, leading to formation of larger (C4b and C2a) and smaller (C4a and C2b) fragments. C4b is able to bind to the target, associate with C2a and form the classical pathway C3 convertase (C4b2a).

The lectin pathway has very much in common with the classical one. It is also initiated by the binding of a protein, mannose binding lectin (MBL) or ficolins (instead of C1q), to specific structures, such carbohydrate or acetylated molecules (instead of Ig Fc receptor). In a similar way as in the classical pathway, serine proteases (called MBL-associated serine proteases or MASPs, instead of C1r and C1s) are activated upon binding to the target, and subsequently activate the complement component C4 and C2 to form the C3 convertase (C4b2a).

The endpoint of the classical and the lectin pathways is the formation of C3 convertase, which can lead to further activation and amplification of the alternative pathway. The convertases involved in the alternative pathway of the complement system are called C3(H2O)Bb and C3bBb. C3(H20) molecules are formed through a spontaneous hydrolysis of C3. C3(H2O) associates with factor B, which is cleaved by factor D into a Bb and Ba fragment. C3 is then cleaved into C3a and C3b. C3b can bind to adjacent surfaces and form complex with factor Bb, generating the alternative pathway C3 convertase. The binding of properdin further stabilizes this complex. Binding to the existing C3 convertase, C3b forms the C5 convertases (C4bC2aC3b and C3bBbC3b) and initiates the common terminal pathway. The C5 convertase cleaves C5 into the small anaphylatoxin C5a and the larger fragment C5b that binds to the cell membrane. The other complement components of the terminal pathway C6, C7 and C8 assemble on the pathogens membrane, where several C9 molecules are incorporated to create cell membrane-penetrating pores.

The complement system has also many regulatory molecules of the activation cascade. Some of them are reported in the figure and will not be further analyzed. Among the inhibitors of the terminal pathway, protein S, or vitronectin, and clusterin inhibit the polymerization and assembly of C9 molecules, respectively. CD59, or protectin, inhibits the formation of the MAC by binding to C8 and C9.

Interaction with complement receptors is needed in many of the immunological functions that the complement factors are involved in.



The classical pathway is triggered by binding of C1q to antibody–antigen complexes. The lectin pathway is similar to the classical pathway, but is activated by binding of MBL to mannose residues, which activates MASP1 and MASP2. The alternative pathway is triggered by spontaneous activation of C3. Activation of these pathways leads to the formation of the MAC—composed of C5b, C6, C7, C8 and many copies of C9—which results in cell lysis. Abbreviations: CFH, factor H; CFI, factor I; DAF, decay accelerating factor; MAC, membrane attack complex; MASP, mannan-binding lectin serine protease; MBL, mannose-binding lectin; MCP, membrane cofactor protein; TAFIa, thrombin-activatable fibrinolysis inhibitor; THBD, thrombomodulin.

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Complement system in SLE

Impairment of the complement system is commonly found in patients affected by SLE.

During disease related flares, patients affected by SLE often display a temporary reduction of complement factors in serum, mostly due to inflammation-related consumption.

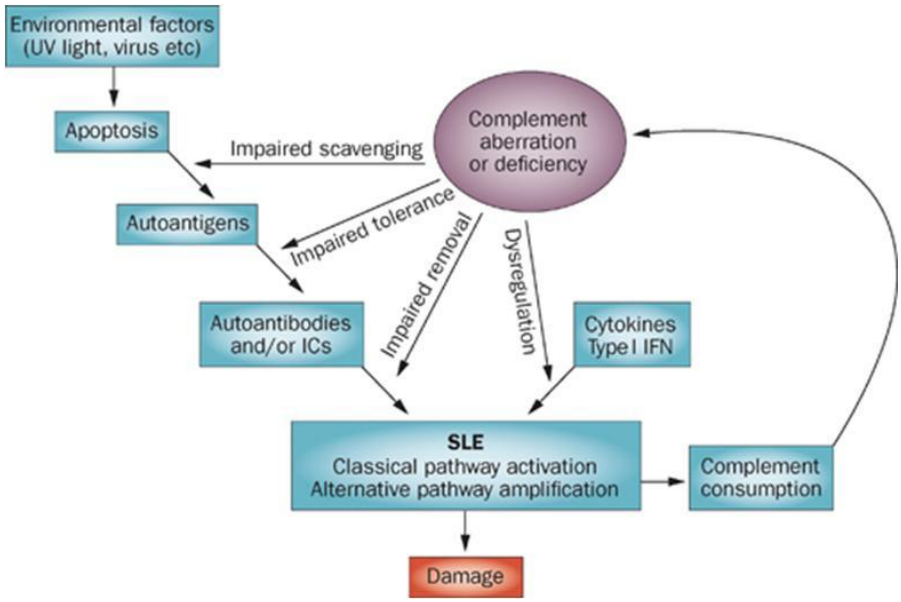
Measurement of serum C3 and C4 has traditionally been included in the "gold standard" for monitoring disease activity in SLE patients. Decreased C3 and C4 levels are considered to be markers of inflammation and increased SLE disease activity. However, the value of serial measurement of serum C3 and C4 in monitoring disease activity in SLE is controversial, since the C3 and C4 serum levels may remain normal during SLE flares and other inflammatory conditions, because activation and consumption are balanced by an increase in C4 and C3 synthesis during acute phase response. Moreover, lower levels of C4 may be due to decreased synthesis rather than increased complement activation and consumption.

In some patients with decreased serum levels of C1q, presence of antibodies to C1q (anti-C1q) is detected. Increased anti-C1q antibodies are strongly associated with lupus nephritis[134 135].

The soluble complement activation products (CAPs) may alter the function of circulating cells by covalently binding (CB) to their surfaces. The assessment of CB-CAPs might serve as more reliable biomarkers than C3 and C4 to guide clinical care of SLE patients.

Complement deficiencies within the classical pathway of activation, but not other deficiencies, pose increased risk of developing SLE, but not autoimmunity in general[136].

The multiple mechanisms by which complement aberration or deficiency may determine damage in SLE patients is schematized in the following figure[136].



Evidence suggests that autoantigens derived from apoptotic cells, and possibly also neutrophil extracellular traps and microparticles, are involved in the generation of autoantibodies that underlie the pathogenesis of SLE. In a genetically predisposed individual, these processes are thought to give rise to a sustained immune reaction resulting in the disease. Deficiency or aberrations in components of the classical pathway of complement activation, either genetically determined or caused by excessive activation and consumption, might be part of the pathogenetic process on multiple levels: impaired scavenging of autoantigens; compromised immune tolerance to self antigens; defective autoantibodies and IC removal; and dysregulated cytokine production. Increased complement activation, as well as causing complement consumption and deficiency, can participate in tissue damage such as glomerulonephritis, which is a hallmark of SLE. Such damage provokes further inflammation and additional complement aberrations, which could contribute to a vicious circle of reactions preventing termination of the disease process. Abbreviations: IC, immune complex; IFN, interferon; SLE, systemic lupus erythematosus; UV, ultraviolet.

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Pattern Recognition Receptors (PRR)

PRR is the general term used to indicate the receptors expressed by different cells, above all scavengers of the innate immune system, specialized in the recognition of virus, parasites, fungi and bacteria. Toll-like receptors (TLR) are the group of 11 trans-membrane proteins, able to recognize the pathogen-associated molecular patterns (PAMPs), conserved structures from different pathogens and to activate the intracellular functions, needed for its immediate destruction. Different TLRs recognize different structures such as lipids (TLR1, 2, 4 and 6), proteins (TLR5 and 10), and nucleic material (TLR9 for DNA, TLR3, 7 and 8 for RNA) in different pathogens, which facilitates the tailoring of the immune response to them.

TLRs have a cytoplasmic tail, to which adaptor proteins (MyD88 or TRIF, TIRAP, TRAM) are recruited, with initiation of different signalling cascades and activation of three main pathways (MAP kinase, NF-kB and IRF), leading to the activation in the nucleus of the transcription of genes involved in the immune response. The TLR stimulation can induce type I IFNs (involved in anti-viral responses), anti-inflammatory (IL-10) or pro-inflammatory (TNF-α, IL-6, IL-12) cytokines, chemokines that attract other immune cells and so on.

Other known PRRs are nucleotide oligomerization receptors (NLR), C-type lectin receptors (CLR) and RIG-1 like receptors (RLR).

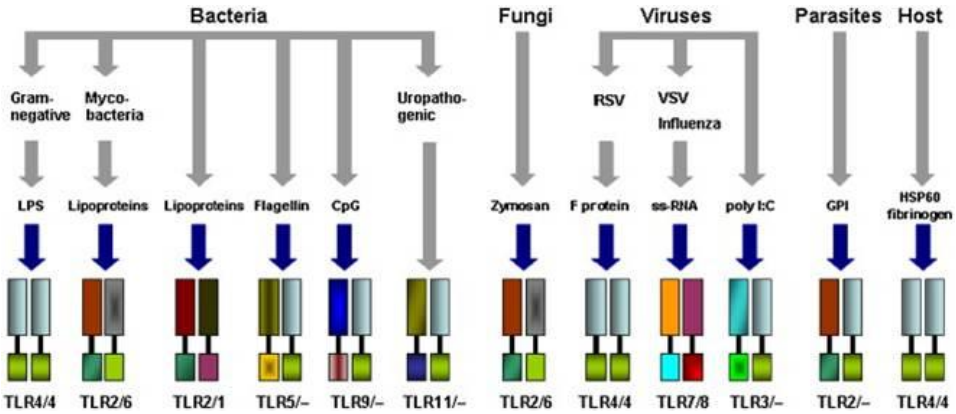


Fig. 1. Ligands of toll-like receptors (TLRs).
TLRs are able to recognize a variety of pathogen-derived products: lipopolysaccharide (LPS) is the ligand for TLR4; bacterial lipoproteins (e.g. lipotechoic acid) are recognized by a TLR2/6 dimer; triacylated lipopeptides by a TLR2/1 dimer; CpG oligonucleotides by TLR9; flagellin by TLR5. TLR11 in mice senses uropathogenic bacteria. A TLR2/6 dimer recognizes zymosan for anti-fungal responses. Anti-viral responses are mediated by TLR4 which senses F protein from RSV, TLR3 which senses double-stranded RNA (poly I:C), TLR7 and TLR8 which sense single-stranded RNA (ss RNA). Protozoal glycosyl-phosphatidyl-inositol (GPI-)anchor proteins are recognized by TLR2. Products of inflamed tissue (e.g. hsp60, fibrinogen products) are sensed by TLR4.

Reprinted from Journal of Autoimmunity, Volume 29, Issue 4, 2007, 310 - 318, Eva D. Papadimitraki,George K. Bertsias,Dimitrios T. Boumpas, Toll like receptors and autoimmunity: A critical appraisal[137], Copyright (2007), with permission from Elsevier

Neutrophils

Neutrophils are the most abundant subset of white blood cells (50–70% of circulating leukocytes) in humans. The main function of the neutrophils consists in eliminating pathogens through phagocytosis, generation of ROS via oxidative burst, degranulation with release of anti-microbial substances, and the release of NETs. Moreover, neutrophils are cells capable of many other specialized functions, contributing to chronic inflammation and adaptive immunity.

Mature neutrophils belong to the so-called polymorphonuclear cells, having segmented nuclei, and have fully formed (primary, secondary and tertiary) granules and secretory vesicles in the cytoplasm. The granules are formed sequentially during maturation. The granules are filled with proteins, many of which are pro-inflammatory. The primary (or azurophilic) ones contain myeloperoxidase (MPO), azurocidin and defensins. The secondary (or specific) granules contain lactoferrin and can be divided into at least four subtypes: lactoferrin_{hi}, $\text{cysteine-rich secretory protein 3 (CRISP3)}_{hi}$, gelatinase_{hi} and ficolin 1_{hi} . The tertiary (or gelatinase) granules contain matrix metalloproteinase 9 (MMP-9) also known as gelatinase B[138].

Neutrophils also contain secretory vesicles that can rapidly be transported and incorporated in the cell surface membrane. Around 50% of neutrophils cytosol consists of S100A8/A9.

The daily production of neutrophils is controlled by the axis granulocyte colony-stimulating factor (G-CSF) – interleukin-17A (IL-17A) – IL-23. Their average circulatory lifespan is up to 5.4 days. During inflammation, neutrophils live longer, increase in tissues and become activated. When neutrophils die by apoptosis and are removed, IL-23 synthesis by macrophages and dendritic cells is down-regulated, which reduces G-CSF release[139].

Neutrophils in SLE

Several abnormalities have been documented during the last years in the function of neutrophils in SLE patients. Serum from SLE patients induces aggregation of normal neutrophils and interference with phagocytosis and release of lysosomial enzymes. SLE-derived neutrophils have impaired phagocytic capacity, enhanced senescence and decreased responsiveness to cytokines. They are activated by several autoantibodies and nucleosomes. Upon activation, they produce and release proteins, such as defensins and lactoferrin, which have been found in serum of SLE patients in increased concentration, in association with increased disease activity. S100A8/A9 is the most abundant protein in neutrophils cytosol and it is released upon cell activation, mostly when NETosis occurs. Increased apoptosis of neutrophils and aberrant clearance of apoptotic material may generate a large amount of autoantigens.

An abnormal subset of neutrophils, referred to as low-density granulocytes (LDG) has been described in the peripheral blood of SLE patients. These cells have high expression of CD15 and low expression of CD14. They express CD10 and CD16, but lack MHC class II and CD86. Increased prevalence of skin and vascular involvement has been documented in patients with high amount of LDG in peripheral blood. LDG have enhanced capacity to undergo NETosis. High levels of LDG correlate with vascular inflammation in SLE patients, suggesting a pathogenic role in the cardiovascular damage. Several studies suggest a role played by neutrophils in SLE related renal manifestations and some types of skin involvement[127].

Monocytes/Macrophages

Monocytes are the largest white blood cells (leukocytes). There are at least three types of monocytes in human blood, differing for expression of CD14 and CD16 on the membrane surface. They circulate in the bloodstream for about one to three days and constitute between 3-8% of the leukocytes in the blood. They migrate later into tissues throughout the body and differentiate into tissue resident macrophages or dendritic cells. Half of them are stored as a reserve in the spleen.

Many factors produced by other cells can regulate the chemotaxis and other functions of monocytes. These factors include most particularly chemokines.

Macrophages are white blood cells that engulf and digest cellular debris, foreign substances, microbes, and cancer cells in a process called phagocytosis. They are found in essentially all tissues. Macrophages that induce inflammation are called M1 or "killer" macrophages and secrete high levels of IL-12, whereas those that decrease inflammation and promote tissue repair by producing IL-10 and TGF-β are called M2 or "repair" macrophages[140].

Phagocytosis, antigen presentation and cytokine production are the main immunologic functions of monocytes and macrophages.

Monocytes can perform phagocytosis using intermediary opsonizing proteins (antibodies or complement) that coat the pathogen or by direct binding to it via PRR. Monocytes are also capable of killing infected host cells via antibody-dependent cell-mediated cytotoxicity (ADCC).

Macrophages are highly specialized in removal of dying or dead cells and cellular debris in strategic locations, such as the lungs, liver, neural tissue, bone, spleen and connective tissue[141].

The pathogen becomes trapped in a phagosome, which then fuses with a lysosome before enzymes and toxic peroxides digest the pathogen. Remaining microbial fragments can serve as antigens that can be incorporated into MHC molecules and then transported to the cell surface of scavengers. This process is called antigen presentation and it leads to activation of T lymphocytes, which then mount a specific immune response against the antigen.

Other microbial products can directly activate monocytes and this leads to production of pro-inflammatory and, with some delay, of anti-inflammatory cytokines, such as TNF-α, IL-1, and IL-12.

Monocytes/Macrophages in SLE

The phagocytic and bactericidal activities of normal monocytes are impaired in the presence of sera from SLE patients with increased clinical activity[142 143]. Reduced clearance of apoptotic cells reflecting phagocyte dysfunction in SLE patients has been observed[16]. Besides clearance defects, monocytes from SLE patients have an abnormal balance in the secretion of anti- and pro-inflammatory cytokines in response to apoptotic cells, not related to disease activity and opsonizing autoantibodies[15]. IFN-α induces the expression of CD64 on monocytes (mCD64). Enhanced mCD64 expression has been reported in patients with SLE, strongly correlated with disease activity[144].

In a recent investigation, the depletion of macrophages in mice resulted in absence of renal damage after administration of rabbit anti-glomerular antibodies, or nephrotoxic serum (NTS), an experimental model which closely mimics the immune complex mediated disease seen in murine and human lupus nephritis. Mice with normal macrophages exhibited significantly increased kidney expression of inflammatory cytokines and development of nephritis, suggesting a strong implication of macrophages in the development of immune mediated glomerulonephritis[145].

Natural killer (NK) cells

NK cells are a type of cytotoxic lymphocyte critical to the innate immune system. They are large granular lymphocytes (LGL), differentiated from the common lymphoid progenitor. They respond rapidly to tumoral cells and viral-infected cells, and recognize stressed cells without mediation of antibodies and MHC, hence resulting in a much faster immune reaction.

NK cells share some phenotypic and functional similarities with (but are not the same as) the natural killer T (NKT) cells, a subset of T cells mostly involved in the adaptive immunity[146].

Natural killer cells in SLE

Decreased numbers of NK cells in the peripheral blood of lupus patients has been reported, but further analyses demonstrated a most likely decrease of the NKT subset of T cells. All patients had fewer than the normal number of circulating NK cells, and other authors found that the numbers of $CD16^{+}CD56^{+}$ NK cells in the peripheral blood of lupus patients were only one-third of levels in control groups. The proportions of NK cells in the peripheral blood were significantly lower in patients with moderate or severe disease, when compared with quiescent disease, and were most depressed in patients with severe lupus nephritis[147 148].

Sequence polymorphisms have been identified in the gene encoding the NK cell marker CD16, which is an Fc receptor for IgG (FcγRIIIa). These polymorphisms affect NK cell function and binding to ligands. Analyses of SLE patients revealed a strong association between homozygosity for one low binding genotype and SLE, especially in patients with nephritis and a very severe numerical deficiency in NK cells[149].

The finding of low killing activity in relatives of SLE patients supports the view that NK cell deficiency is a genetic determinant of SLE. NK cells in SLE may produce insufficient levels of cytokines required for the regulation of IgG production[150].

Dendritic cells

The dendritic cells (DCs) are sentinel cells that bridge the innate and adaptive immune systems. DCs recognize pathogens using PRRs, mostly TLRs, and then migrate to lymphoid organs to present pathogen-derived antigens to antigen-specific T cells. DCs comprise the conventional or classical DCs (cDCs), the plasmacytoid DCs (pDCs), the Langerhans cells and the monocyte derived DCs.

The cDCs are antigen-presenting cells (APCs) with a characteristic dendritic morphology, expressing high levels of MHC class II molecules. A subset of cDCs is $CD8^+$, which can mediate antigen cross-presentation to cytotoxic $CD8^+$ T cells. Another subset is $CD11b^+$, which preferentially presents antigens to $CD4^+$ T cells. The pDCs rapidly produce type 1 interferon (IFN) following activation through TLRs. In lymphoid organs and tissues, there might be some functional division within each subset into 'presenter' and 'detector' DCs.

Depending on the inflammatory context and the expression of regulators, DC-mediated presentation of self-antigens might promote or inhibit autoimmune responses. Activated DCs up-regulate co-stimulatory molecules and produce cytokines that drive T cell priming and effector differentiation, and they activate various types of immune cells. In absence of activation, antigen presentation by steady-state DCs might lead to T cell unresponsiveness and might promote tolerance[18].

Dendritic cells in SLE

Experimental studies have shown that the loss of CD95 (or FAS) in murine DCs induces lupus-like manifestations, whereas the constitutive deletion of DCs in SLE-prone mice ameliorates the clinical features[151 152].

BLIMP1 controls the differentiation of B and T cells. Polymorphisms in the gene PRDM1, encoding BLIMP1, have been described in SLE[153]. BLIMP1-deficient DCs induce increased IL-6 production leading to differentiation of T_{FH} cells, with enhanced humoral autoreactivity, especially in females[154].

The important role played by type I IFNs in SLE pathogenesis draw the attention to the pDCs as potential source of the cytokine. Immune complexes containing nucleic acids can activate pDCs and induce secretion of type I IFN, as seen in NETosis. It activates monocytes and neutrophils, which implement the inflammatory response in a vicious circle[11 132].

Antibodies or immunoglobulins

Immunoglobulins (Ig) are large molecules produced by mature B-cells, called plasma cells, and consist of two heavy and two light protein chains. The heavy chains are composed by three constant domains (C_H1, 2 and 3) and one variable domain (V_H). The light chains consist of a constant domain (C_L) and a variable domain (V_L). Two different fragment (F) regions are formed by the combination of the chains, referred to as Fab (antigen binding) region and Fc (crystallisable) region. Ig are Y-shaped, being the Fab regions the "arms" and the Fc region the "tail". The Fab regions attach any complimentary binding site present in antigens, through six different loops, referred to as complementary determining regions (CDR). The Fc region binds to receptors present in cells of the innate immunity.

The main function of Ig is to stop the invasion of pathogens, by different mechanisms. Opsonization is the mechanism by which antibodies link antigens (bound via Fab regions) to scavengers (bound via Fc region), facilitating the engulfment and destruction of the pathogens.

Binding antigens lead to formation of ICs that activate the destruction of the pathogens after activation of the complement system.

Five different classes of Ig, differing for the constant domains and functions, are known: IgA, IgD, IgE, IgG and IgM. Originally, only IgD and IgM are expressed by B-cells. Upon maturation, according to stimulation they get by the environment and cytokines, the B-cells can switch to other Ig-classes. IgG are the largest amount of Ig in blood and 4 different subclasses have been described.

Antibodies in SLE

Antibodies in general and autoantibodies in particular, play a pivotal role in the pathogenesis of SLE. A plethora of autoantibodies have been described in biologic samples analyzed in patients affected by SLE.

Up to more than 95% of SLE patients have high serum levels of antinuclear antibodies (ANA). Although many other autoantibodies exist, their precise role in pathogenesis has not fully been elucidated. Histological analyses of kidneys and skin in SLE demonstrate inflammation and deposition of both complement and Ig, herein autoantibodies.

Besides anti-dsDNA antibodies that will be further analyzed in a separate paragraph, autoantibodies anti-Ro (ribonucleoprotein complex), anti-La (RNA-binding protein), anti-Sm (nuclear particle with several different polypeptides) and anti-C1q have been described in renal biopsies of SLE patients. It is still unknown whether these antibodies are directly pathogenic or not, and exactly by which mechanism they determine tissue destruction. One plausible explanation could be the formation of immune complexes with circulating autoantigens, for example nucleosomes released by apoptotic cells, with following deposition on target tissues and inflammatory response, mediated by the activation of the complement system. An alternative mechanism could be the direct binding of circulating autoantibodies, due to cross-reactivity, to proteins in the target tissue.

Specific associations of autoantibodies with particular clinical features of SLE are postulated, such as anti-C1q antibodies with glomerulonephritis[134], anti-Ro antibodies with cutaneous lupus[155] and anti-NMDA antibodies with CNS lupus[156].

Lymphocytes

The lymphocytes are a subset of leukocytes, or white blood cells (WBC). About 20-40% of the WBC present in the human body (mostly tissues and lymphatic system) and 2% of the circulating cells in the peripheral blood are lymphocytes. They are produced by stem cells in the bone marrow and then differentiate and migrate during their maturation.

B cells and T cells are the major types of lymphocytes. B cells are involved in the humoral immune response, whereas T cells are responsible of the cell-mediated acquired immunity. The names B and T derive from the main site where the cells maturation was discovered to occur, namely the bursa of Fabricius in birds (bone marrow in mammals) and the thymus.

An accurate early selection of lymphocytes occurs in the bone marrow and the thymus, where the maturation of highly reactive cells to self is stopped, through a mechanism called central tolerance.

Millions of **naïve B cells**, each with a unique antigen-specific protein on the surface, called B cell receptor (BCR), are produced every day to guarantee the immune surveillance. When they encounter and bind their specific antigen, the B cells are cloned, with production of many functional (or **effector**) B cells that undergo further differentiation steps. The majority becomes **plasma cells**, large short-lived cells that produce and secrete specific antibodies against the antigen that stimulated the proliferation. When the antigen is eliminated, the plasma cells undergo apoptosis. A minor amount of effector B cells will differentiate into **memory B cells**, long-lived cells responsible of the secondary immune response, where the B cells maturate their affinity to the particular antigen and can respond quickly when exposed to it in the future.

The activation of B cells can occur with or without the involvement of T cells. Most antigens need to be processed and presented by the B cells to a subset of the T cells (called T helper or T_H) that secretes activating molecules, called cytokines, promoting the differentiation of B cells into plasma cells and the subsequent production of antibodies, type IgM or IgD.

Mature B cells migrate to the germinal centers within the peripheral lymphoid organs (lymph nodes and spleen) where the Ig isotype switching to IgG, IgA and IgE takes place.

The B cells can be also activated by TLR, resulting in exclusive production of IgM against the TLR-binding antigen.

The **T cells** are distinguished from other lymphocytes for the presence of a T cell receptor (TCR). Depending on expression or absence on cell surface of different chains composing the TCR and of other co-receptors, such as CD4 and CD8, during the maturation process, the T cells can be divided into different arbitrary subsets.

All the T cells are TCR^- $CD4^-$ $CD8^-$ in a very early phase of the development. A small amount of T cells maintain the mentioned double negativity ($CD4^-$ $CD8^-$) in a mature stage, after expressing the TCR. As they progress through the maturation in the thymus, T cells become double positive ($CD4^+$ $CD8^+$) and undergo a positive selection. Only the T cells surviving the selection maturate into single positive T cells, because of down-regulating expression of one co-receptor.

$CD4^+$ T cells are called **T helper (T_H)**, since they assist other cells in immunological processes. Depending on which cytokine they secrete once activated, the T_H cells can differentiate into different subtypes, such as T_{H1} (producing IFN-γ), $T_{H\,2}$ (producing IL-4) and so on. The T_{FH} cells are the $CD4^+$ T cells found in germinal centers in the peripheral lymphoid organs, where they encounter and interact with the follicular B cells, and instruct them to undergo differentiation into plasma cells and memory B cells.

$CD8^+$ T cells are called **cytotoxic T cells (CTL or T_c)**, since they destroy non-self cells. Likewise the B cells, the T cells also have a long-lived subset, either $CD4^+$ or $CD8^+$, called **memory T cells**, typically expressing the protein CD45RO on the surface.

A subpopulation of T cells that modulates tolerance to self-antigens is the **regulatory T cells (T_{Reg})**, formerly known as suppressor T cells. These cells shut down the immune response when the invading organism has been eliminated and prevent the risk of self-reactivity.

A subset of T helper, referred to as **T_H17 cells** produce IL-17, involved in the pathogenesis of many inflammatory and autoimmune diseases and are regulated by the so called **T_{Reg} 17 cells**.

The **natural killer T (NKT) cells** represent another subset of T cells, able to produce IFN-γ and IL-4 when activated.

Lymphocytes in SLE

Multiple B cell abnormalities have been reported in SLE[157], which facilitate the aberrant immune response and result in the formation of antibodies targeting self-antigens. It is not clear if these dysfunctions in B cells are the result of defects at a central "checkpoint" level or arise in the periphery from abnormal selection.

There is some evidence to suggest that defects in early negative selection exist, resulting in the persistence of immature phenotype in naïve B cells, which may facilitate self-reactivity.

The repertoire of circulating B cells is also altered in SLE with a skewing toward increased frequencies of pre-immune B cells, memory cells, and plasma cells.

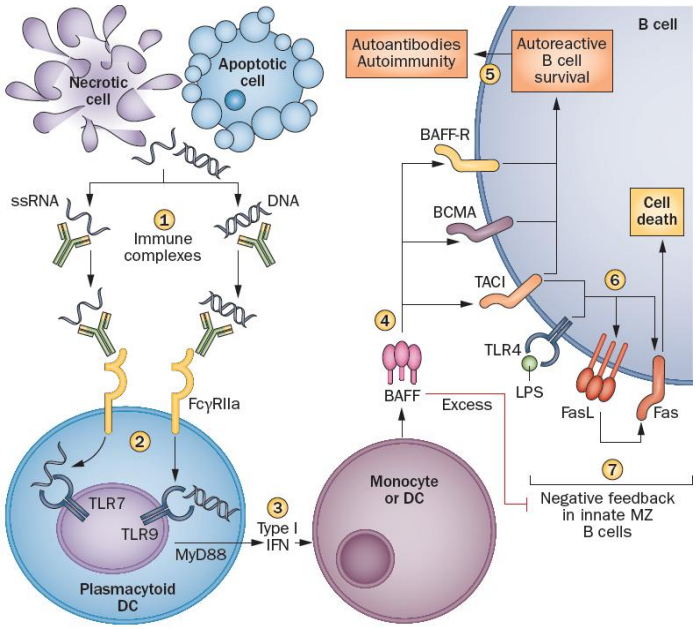
The circulating memory cells are less responsive to immune suppression and can be activated rapidly by TLR agonists (such as trans-membrane activator and calcium modulator and cyclophilin ligand interactor, or TACI)[158 159] or cytokine combinations, independent of antigen or T cells.

The ratio $CD27^+$ plasma cells/ $CD27^+$ plasmablasts is lower in SLE, usually associated with increased disease activity[160]. Increased amount of antigen-specific memory B cells and Ig-secreting plasma cells has been reported[157]. A pathogenic role of the long-lived plasma cells in SLE has been proposed[161].

The known association between SLE susceptibility and polymorphisms in the genes encoding FcγRIIB and BLIMP-1 further highlights the importance of B cells in SLE pathogenesis[162-164].

The regulatory B cell population has been shown to lack full functionality in SLE. These cells can secrete IL-10 and can suppress T_H1 and T_H2 functions[165].

The role played by the BAFF/APRIL system in SLE pathogenesis is schematized in the following figure[166].



Role of BAFF in the pathogenesis of SLE. In SLE, it is believed that antibody–nucleic acid immune complexes (1), for example ssRNA or DNA from dead cells, are bound by FcγRIIa, activating TLRs and IFN‑α production (2). IFN‑α increases BAFF production (3). BAFF interacts with receptors on B cells (4). Excess BAFF can increase autoreactive B‑cell survival, driving autoimmunity (5). TLR4 and TACI signalling cooperate to commit MZ B cells to apoptosis via induction of Fas and FasL (6), possibly contributing to the mechanism that terminates the short-lived antibody response of activated innate B cells. This mechanism is defective in BAFF-overexpressing transgenic mice (7). Abbreviations: BAFF, B‑cell-activating factor of the TNF family (also known as TNF ligand superfamily member 13B); BAFF‑R, BAFF receptor (also known as TNF receptor superfamily member 13C); BCMA, B‑cell maturation antigen (also known as TNF receptor superfamily member 17); DC, dendritic cell; FasL, Fas ligand; FcγRIIa, immunoglobulin γ Fc region receptor IIa; LPS, lipopolysaccharide;MyD88, myeloid differentiation primary response protein MyD88; MZ, marginal zone; ssRNA, single-stranded RNA; TACI, transmembrane activator and cyclophilin ligand interactor (also known as TNF receptor superfamily member 13B); TLR4, Toll-like receptor 4.

Reprinted by permission from Macmillan Publishers Ltd: Fabien B. Vincent, Eric F. Morand, Pascal Schneider, Fabienne Mackay. The BAFF/APRIL system in SLE pathogenesis. Nature Reviews Rheumatology 10, 365–373 doi:10.1038/nrrheum.2014.33, copyright 2014[166]

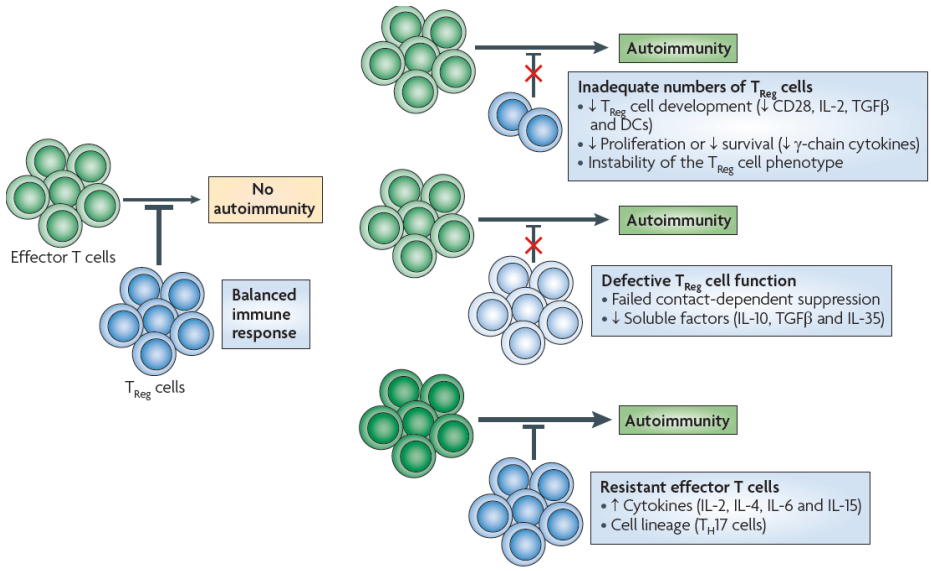
Abnormalities in various subsets of T cells have been reported in autoimmunity, although the mechanisms and functional consequences of such changes remain unclear.

SLE is traditionally described as a T_H2-mediated disease, but it is now known that T_H17 and T_{Reg} cells are also of importance. Patients with SLE have increased numbers of T_H17 cells and elevated IL-17 levels in the serum and the kidneys. It is probably the effect of enhanced activity of the IL-17 promoter, mediated by a

combination of intra-cellular factors, such as ROCK (rho-associated protein kinase), and CREM (cAMP-responsive element modulator) alpha, which are increased in SLE patients[167 168].

T follicular helper (T_{FH}) cells express CXCR5. A subset of SLE patients was found to have increased levels of $CD4^+CXCR5^+$ T cells [169], cells that mimic T_{FH} and lead to increased autoantibody production and tissue damage in SLE.

T regulatory (T_{Reg}) cells are $CD4^+$ T cells characterized by the expression of the transcription factor Foxp3 (forkhead box p3). It was demonstrated that $CD4^+Foxp3^+$ T cells are decreased in patients with SLE, which contributes to lack of self-tolerance. There are also reports that effector T cells in SLE patients may be resistant to the effect of T_{Reg} cells [170]. In SLE, T_{Reg} cells may also have a greater susceptibility to cell-mediated death. Moreover, effector T cells may be more resistant to the suppressive effect of T_{Reg} cells, independently of disease activity.



Causes of impaired T_{Reg} cell-mediated suppression in autoimmunity. Autoimmunity can result from a loss of regulation of autoreactive T cells. Failures of regulatory T (T_{Reg}) cell-mediated regulation include: inadequate numbers of T_{Reg} cells owing to their inadequate development, proliferation or survival; defects in T_{Reg} cell function that is intrinsic to T_{Reg} cells; and resistance of pathogenic effector T cells to suppression by T_{Reg} cells owing to factors that are intrinsic to the effector cells or factors that are present in the inflammatory milieu and that support effector T cell resistance. DC, dendritic cell; IL, interleukin; TGFβ, transforming growth factor-β; TH17, T helper 17.

Reprinted by permission from Macmillan Publishers Ltd: [Nature Reviews Immunology 10, 849-859 | doi:10.1038/nri2889 Mechanisms of impaired regulation by $D4^+CD25^+FOXP3^+$ regulatory T cells in human autoimmune diseases Jane Hoyt Buckner (reference citation), copyright 2010[171]

Cytokines

"Cytokines" (from Greek "cell movement") is the term used to categorize many small proteins involved in different ways in cell signalling. Cytokines are often considered as the means by which cells communicate and interact with the environment. Cytokines are pleiotropic proteins produced by a broad range of cells, including immune cells. They can be classified in different ways, according to structure or function and so on. According to the National Library of Medicine - Medical Subject Headings (MeSH), the current list of the cytokines consists of the following subsets: Chemokines, Interferons, Interleukins, Lymphokines, Autocrine Motility Factor, Growth Differentiation Factor 15, Hematopoietic Cell Growth Factors, Hepatocyte Growth Factor, Interleukin 1 Receptor Antagonist Protein, Leukemia Inhibitory Factor, Oncostatin M, Osteopontin, Transforming Growth Factor β and tumour necrosis factor (TNF). Their binding to receptors modulates and regulates different important cell functions. Chemokines mediate attraction (chemotaxis) between cells. Interferons, are involved in antiviral responses. Interleukins are mainly produced by T-helper cells. Lymphokines are produced by lymphocytes. Monokines are produced exclusively by monocytes. Osteopontin will be discussed separately in this thesis.

Cytokines involved in immunological processes are commonly divided into type 1, which favor cell-mediated immune responses, such as IFN-γ and TNFα, and type 2, enhancing humoral responses, such as TGF-β, IL-4, IL-10, IL-13.

Cytokines in SLE

A number of cytokines are up-regulated in SLE and have been proposed as potential biomarkers or therapeutic targets.

The serum levels of type 1 IFN and of IL-6 are increased in patients with SLE and correlate with both disease activity and anti-dsDNA titers.

Many cells can produce **IFN-α** in response to viral infection in small quantities. Plasmocytoid DCs, previously called 'naturally interferon-producing cells', are unique in their capacity to produce vast amounts of IFN-α capable of generating systemic effects. More than half of SLE patients show increased circulating type I interferon in association with clinical manifestations of disease[172]. Further description follows in the chapter dedicated to biomarkers in SLE.

IL-6 is primarily secreted by monocytes, endothelial cells and fibroblasts. In SLE patients, increased IL-6 in urine[173] and enhanced IL-6 expression in glomeruli and

tubules have been associated with nephritis[174], as well as increased IL-6 in liquor has been documented in patients with CNS lupus[175 176]. The preliminary results of treatment with monoclonal antibody anti-IL-6 (Tocilizumab) in a limited number of SLE patients are encouraging[177]. Phase I trials indicate that Sirukumab, a monoclonal antibody with high affinity for IL-6, is generally safe and well tolerated and preliminary data show improvement in patient-reported outcomes, and transient improvement in clinical parameters[178].

APRIL is the acronym for a proliferation-inducing ligand, a cytokine that activates B-cells. It shares binding to the same receptors and many of the biological functions with BLyS, which is essential in the survival of B cells and is a powerful stimulator of B cell proliferation and immunoglobulin secretion. Serum levels of BLyS and APRIL are elevated in SLE patients and correlate with anti-dsDNA antibodies. Belimumab, a fully humanized monoclonal antibody that binds soluble BLyS, is now an approved and licensed treatment for SLE. Atacicept is a fusion protein containing the extracellular, ligand-binding portion of the receptor TACI and the modified Fc portion of human IgG that blocks BLyS (like Belimumab) and APRIL. The dual blockade by Atacicept may be more potent than blockading BLyS alone and has the benefit of targeting long-lived plasma cells in addition to B cells. Preliminary data from clinical trials using Atacicept are somewhat controversial, showing the efficacy of the drug, along with reported severe adverse events[179].

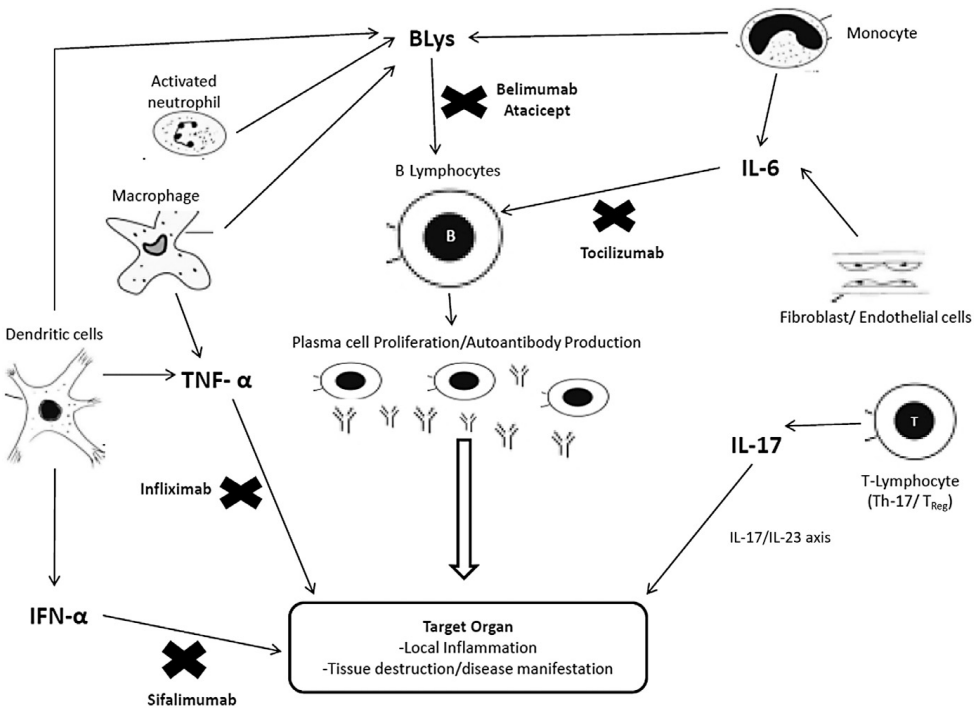
Tumor necrosis factor-alpha **(TNF-α)** is expressed as a trimer on the cell surface and in soluble form after the activation of macrophages and dendritic cells.

The significance of TNF-α in the pathogenesis of SLE remains controversial since it has been depicted as both protective and detrimental in different murine models[180 181]. Serum levels of TNF-α in active SLE patients closely correlated with disease activity[182]. High TNF-α mRNA is found in renal biopsy specimens from patients with LN[174]. Positive effect of the TNF inhibitor Infliximab in patients with SLE is documented, but patients with chronic inflammatory diseases receiving anti-TNF-α therapy may develop ANA, anti-ds DNA and aCL antibodies as well as a lupus-like syndrome that usually resolves following discontinuation of TNF-α blocking therapy[183].

IL-10 is a monocyte- and lymphocyte-derived cytokine with both pro- and anti-inflammatory roles. It impedes the activation of antigen presenting cells (APC), blunts T cell activation and TNF-α secretion, boosts B cell proliferation and immunoglobulin class switching resulting in enhanced antibody secretion. Anti-ds DNA antibodies and immune complexes bound to FcγRII are potent triggers of IL-10. Early clinical studies on its inhibition have shown some improvement in patients with SLE, primarily in cutaneous and joint symptoms[181].

IL-17, IL-21 and **IL-22** are cytokines produced by T_H17 cells. IL-17 is a potent pro-inflammatory type I transmembrane protein. Serum levels of IL-17 are increased in patients with SLE and correlate with disease activity[184 185].

Daily injections of **IL-18,** with or without **IL-12,** resulted in accelerated proteinuria, glomerulonephritis, vasculitis and elevated levels of pro-inflammatory cytokines in MRL/lpr mice[186]. Serum IL-18 levels were higher than in controls in SLE patients and were correlated with urinary microalbumin[187 188]. Several studies have described that the IL-18 levels correlate with the anti-dsDNA titers and the SLEDAI score. IL-18 was abundantly expressed in biopsy samples of lesional skin from patients with cutaneous lupus. Kahlenberg et al. have recently demonstrated that inflammasome activation of IL-18 would result in endothelial progenitor cell (EPC) dysfunction, which might explain premature atherosclerosis in SLE patients[189 190].



Schematic diagram to illustrate the complex interaction of different cytokines/immune cells and the rationale of anti-cytokine therapies.

IL, interleukin; TNF-α, tumour necrosis factor-α.
From: Yap, D. Y. H. and Lai, K. N. (2013), The role of cytokines in the pathogenesis of systemic lupus erythematosus – from bench to bedside. Nephrology, 18: 243–255. doi: 10.1111/nep.12047[189]

Biomarkers

A working group within the National Institutes of Health (NIH) in USA defined a biomarker as a ‘‘characteristic that is objectively measured and evaluated as an indicator of normal biological processes, pathogenic processes or pharmacologic responses to a therapeutic intervention’’[191].

“Biomarker” and “surrogate endpoint” are often used as synonymous, but it is important to point out that the requirements for surrogate endpoint are more stringent, as it has to be a “validated measurement that is intended to serve as a substitute for a clinically meaningful outcome and is expected to predict the effect of a therapeutic intervention”[191].

Biomarkers may assess the risk of developing a disease (antecedent biomarkers), identify individuals with subclinical disease (screening biomarkers), aid in diagnosis of overt disease (diagnostic biomarkers), estimate disease severity (staging biomarkers) and provide information on the course of a disease, predict response to therapy, or monitor efficacy of a therapeutic strategy (prognostic biomarkers)[191].

The use of biomarkers is common in clinical settings to early screen patients, to diagnose, to assess the activity of disease, to get prognostic information and to guide therapy.

The ideal biomarker should measure a clinically relevant process, needs to be accurate, to show good sensitivity (identifying disease when it is present) and specificity (excluding disease when it is not present), to be relatively non-invasive and be reliably reproducible. For use in clinical practice, it should also demonstrate cost- effectiveness.

A biomarker must go through several phases of development, including planning, discovery, validation and commercialization. Biomarker discovery usually begins with the **identification** of a proposed biomarker through techniques such as proteomics, metabolomics and transcriptomics. This is generally followed by **cross- sectional** analysis of the clinical utility of the proposed biomarker to predict a firm outcome. Biologic samples (serum, plasma, urine, synovial fluid, liquor etc.) from two or more groups of subjects are analyzed and compared to look for significant differences.

The candidate biomarker needs to demonstrate its sensitivity and specificity using receiver- operating characteristic (ROC) curves to determine the optimal biomarker concentration that achieves the most reliable interpretation. Following cross-sectional assessment, proposed biomarkers need to demonstrate an ongoing relationship with standard disease markers, with fluctuations in disease activity and/or with interventional processes over a defined **longitudinal** period.

Validation means that a biomarker accurately and reproducibly measures what it is supposed to measure. **Qualification** indicates that the biomarker measures something that can predict a clinical outcome and can be considered as surrogate endpoint[192].

Biomarkers in SLE

Being SLE a multifaceted systemic disease, there is no clearly standardized or validated "gold standard" biomarker that covers all the aspects and the different phenotypes of the disease. Traditional laboratory assays used for diagnosis and monitoring of SLE, such as anti-nuclear antibodies (ANA), antibodies against the double-stranded DNA (anti-dsDNA) and complement factors in serum, have never been standardized or validated themselves. Although anti-dsDNA antibodies are highly specific for SLE, the majority of patients are sometimes negative for anti-dsDNA during the disease course. It is one of the main topics of this thesis, described in details in a separate chapter.

Biomarkers could be used in SLE for several purposes, such as screening, prediction of risk in individuals or populations, to establish or confirm the diagnosis, to monitor the disease course, activity and severity, to provide prognostic information regarding future organ involvement, response to therapy, risk of complications, co-morbidity and mortality.

The lack of fully reliable biomarkers affects diverse aspects of the approach to SLE patients. Combining typical clinical features with specific laboratory findings is the best way to make the diagnosis of SLE. Diseases that may mimic SLE must concomitantly be ruled out. There is no laboratory test either with reliable capacity to predict a disease flare or an organ involvement. Moreover, the discovery of good biomarkers involved in the SLE pathogenesis could be crucial in development of new treatments (targeting the biomarker itself or related molecular and cellular mechanisms) and would facilitate the proper selection of subsets of patients and the assessment of their response to treatment.

An increasing amount of novel SLE biomarkers are proposed, based upon clinical and/or laboratory investigations, but very few biomarkers are later validated and

even fewer, if any, will ever fulfill the requirements as surrogate endpoint. For many molecules, significant differences are found between SLE patients and healthy people or other control groups, but none of these molecules has later been validated as a specific diagnostic biomarker of SLE. It is partially due to the fact that the underlying pathophysiologic processes are obscure and complex and some biomarkers that are useful at one particular stage of the disease may not be useful at other stages. Moreover, the misleading results in some clinical investigations are often influenced by the study design (cross-sectional vs longitudinal), by the selection and inclusion of cases, or by the choice of control groups and outcome measures. The effort itself to evaluate all the SLE patients as a single group is probably misleading, so that any biomarker that might be highly valuable in a certain subset of patients could result fully indifferent in another subset or in the joint analysis of mixed clinical phenotypes. In addition, when patients are included in studies searching for novel biomarkers, the inclusion is often based on SLE classification criteria, with a concrete risk of inclusion of subjects who actually do not have the disease. The ones who in fact have SLE may compose a heterogeneous group of patients anyway, because of different therapies, or stages of disease, or ethnicity, all variables that may be crucial to determine the clinical relevance of any potential biomarker. Furthermore, many promising biomarkers found in animal models of human diseases do not perform well in humans, because of any interspecies differences. It is not uncommon that the methods used to evaluate novel biomarkers are not standardized and controversial results between laboratories can be obtained. A final point to emphasize is that misleading information about potential biomarkers may generate from investigators who tend to draw conclusions only based on the statistic significance of the results, which anyway may lack the power needed to extrapolate those results to the whole population of patients affected by the disease.

Sensitivity, specificity, precision, reproducibility and probability of false-positive and false negative results should be validated[192].

Nowadays, autoantibodies represent the classical hallmark of SLE, as they are central in the diagnostic process and in the management of all the SLE patients. False-positive and false-negative results are frequent and a plethora of new (more or less validated) methods are used for the assessment of the same antibodies, with the assumption to be as valid as the traditional assays.

A few out of the hundreds suggested novel biomarkers draw the attention to interesting aspects of the disease. A detailed analysis of the most promising biomarkers has recently been reported by Ahearn and colleagues[193], who attempted a classification of SLE biomarkers, distinguishing biomarkers for lupus susceptibility from those for diagnosis, for disease activity and for specific organ involvement.

Biomarkers for susceptibility

Initial genetic studies focused on genes that are historically considered to be key components of immune responses, such as major histocompatibility complex (MHC) and human leukocyte antigen (HLA) genes[47 194 195]. Currently, the genes that most warrant being referred to as ''lupus genes'' are those encoding the components of the classical complement pathway. Two different genes, each with 0-4 copies, encode the 2 isotypes of human C4 **(C4A and C4B)**, giving a copy number variation (CNV) ranging from 0 to 8 copies. SLE susceptibility is significantly increased among individuals with only 2 copies of the C4 genes (particularly C4A), but is decreased in those with 5 or more copies[196].

Recent genetic studies of SLE have focused on correlating SLE with **polymorphisms** of hypothetical candidate genes coding for mannose-binding lectin (MBL)[197 198], cytokines (IL-1[199], IL-10[200], IL18[201], IL-21[202], TNF-α[203], and Osteopontin[204]), chemokines (MCP-1[205]), cytokine receptors/antagonists (type II TNF-α receptor[206] and IL-1 receptor antagonist[207]), Fcγ receptors (FcγRIIa[208], FcγRIIb[162], FcγRIIIa[209], and FcγRIIIb[210]), and other cell surface receptors (cytotoxic T lymphocyte antigen-4 or CTLA-4[211] and programmed death protein-1 also known as PD-1 or PDCD-1)[212].

Additionally, **CNV** of some candidate genes have been investigated as susceptibility factors. Low CNV or complete deficiency of FcγRIIIb renders an individual at considerably increased risk for autoimmune diseases, particularly SLE[210].

Aberrant T and B cell functions are hallmarks of immune abnormalities in SLE. Protein tyrosine phosphatase N22 **(PTPN22)** is a lymphoid-specific enzyme regulating TCR signalling in memory/effector T cells. It was found that a single nucleotide mutation in PTPN22 is strongly associated with SLE in Caucasian patients, especially in familial SLE of North American patients with European ancestry and in patients with SLE and concurrent autoimmune thyroid disease. Other mutations have been suggested to be relevant for susceptibility in SLE patients with other ethnicity[43].

As mentioned, high serum levels of IFNα have long been detected in lupus patients. Molecules involved in the regulation or execution of the IFNα-pathway have been studied in the search for lupus biomarkers. IFN regulatory factor 5 **(IRF5)**, a transcription factor that controls trans-activation of IFNα-related genes, has emerged as the leading contender. Likewise PTPN22, genetic variants of IRF5 have also been reported to confer risk for non-SLE autoimmune diseases.[42 45 213-215]

The signal transduction and activator of transcription 4 **(STAT4)** gene encodes a transcription factor involved in the signalling pathways of several cytokines, including IL-12, IL-23, and type I IFN. Haplotype with a specific polymorphism

within the third intron of the STAT4 gene is significantly associated with increased risk for both RA and SLE and other autoimmune diseases[44-46].

Genes encoding C-reactive protein (CRP)[216 217], pre-B cell leukemia transcription factor (PBX1)[218], polyADP-ribose polymerase (PARP)[219], B lymphoid tyrosine kinase (BLK)[45], integrin aM (ITGAM; CD11b)[220], and integrin aX (ITGAX)[221] have been identified as susceptibility loci by genome-wide linkage analyses, but still await further search and validation.

Biomarkers for diagnosis

Determination of autoantibodies directed to nucleic acids, nucleosomes and other nuclear components is commonly used in diagnosing and monitoring SLE.

Presence of **antinuclear antibodies (ANA)** in the blood/serum is a hallmark of SLE, used in diagnosis and in monitoring of disease progression. The detection in serum of high ANA titer represents one of the classification criteria for SLE[60-63].

The antigens responsible of the reactivity of ANA are mostly DNA, histone proteins and other extractable nuclear antigens (ENA), such as Ro/SSA, La/SSB, snRNP, Sm, and many others. However, there are considerable negative aspects related to the use of these immunologic markers[193], consistent with some of the results[222] shown in this thesis and with previous reports[223].

Many of these autoantibodies have been also associated with other autoimmune rheumatic diseases, such as Sjögren´s syndrome, systemic sclerosis, dermatomyositis and polymyositis, besides SLE. ANA may also be seen in patients with non-rheumatic diseases, especially autoimmune diseases, but also during current infections and malignancies. Up to 5% of healthy individuals may be ANA positive, especially women and elderly people.

So far, detection of ANA using indirect immunofluorescence (IF-ANA) is considered as the "gold standard" screening test. Due to its low specificity, it is recommended that all IF-ANA- positive samples should be analyzed further for more specific autoantibodies, i.e., dsDNA and ENA. IF-ANA is cheaper, relatively easy to perform, and has good sensitivity. Nowadays, most of the labs use cultured human epithelial cells of laryngeal squamous cell carcinoma (Hep-2) as standard substrate for IF-ANA. Nearly all clinically relevant ANA react to IgG irrespective of the presence of IgM and IgA, while in healthy individuals IF-ANA positivity is usually due to IgM and IgA only. The interpretation of IF-ANA requires skill and experience. The intensity is evaluated in a semi-quantitative manner, along with the assessment of the pattern of fluorescence (homogeneous, peripheral, speckled, nucleolar and centromeric) and the titer of ANA, in case of positive result.

It is getting more and more common to measure ANA by ELISA, providing a quantitative measurement, independently of the skills of the one assessing the results, but missing the features concerning the pattern of fluorescence, which in the past has been associated to specific autoimmune rheumatic diseases.

Antibodies to nucleosomes and histones are frequently reported in patients affected by SLE. Nucleosomes are the basic subunit of chromatin. They consist of DNA wrapped around a histone octamer that is made up of 2 copies each of the core histones H2A, H2B, H3, and H4. The linker histone H1 binds the nucleosome and locks the DNA into place.

Anti-nucleosome antibodies are present in 70% to 100% of patients with SLE and have a high specificity (up to 97%) for the disease. Among SLE patients, antinucleosome antibodies are more prevalent in patients with nephritis and may serve as useful biomarker in the diagnosis of active lupus nephritis, along with a strong correlation with SLE disease activity.

Anti-histone antibodies are detected in 75% of patients affected by drug-induced lupus and in up to 75% of the cases of idiopathic SLE. In particular, IgG anti-(H2A-H2B)-DNA complex are found in 90% of the cases of procainamide-induced lupus, less commonly with other drugs. The IgG type of these Abs, but not IgM or IgA when found, should point to drug induced lupus[224]. The clinical utility of anti-histone antibodies assessed by ELISA to diagnose SLE is considered lower than other traditional antibodies[225].

Although **anti-C1q antibodies** can be detected in a small proportion of healthy individuals (2%–8%), they are more common in patients with autoimmune disorders such as hypocomplementemic urticarial vasculitis and in 30% to 60% of patients with SLE. Strong correlation between the presence of anti-C1q antibodies and renal involvement in SLE has been reported. The absence of anti-C1q antibodies has been reported to exclude a diagnosis of lupus nephritis and an increase in anti-C1q antibodies has been suggested to predict renal flares. Levels of anti-C1q antibodies decreased after successful treatment of lupus nephritis[134].

Abnormal levels of erythrocyte-bound complement activation product C4d **(E-C4d)** and complement receptor 1 **(E-CR1)** have been proposed as reliable candidate biomarkers. Patients with SLE have higher E-C4d and lower E-CR1 levels than controls. The **E-C4d/E-CR1 test** was shown to be highly sensitive and specific for SLE and often abnormal already at an early stage of disease[226]. Deposition of C4d on lupus erythrocytes may also participate in the pathogenesis of the disease[227].

P-C4d are the complement activation products (CAPs) bound on platelets.They are also a potential biomarker for lupus diagnosis, being 98-100% specific in SLE patients[228].

Lymphocyte-bound CAPs, both **T-C4d and B-C4d**, displayed sensitivity around 60% and specificity around 80% in differentiating SLE from other diseases[229].

Biomarkers for disease activity

As previously mentioned, disease activity in SLE is often assessed using standardized composite disease activity indices[67], which comprise a variety of clinical and laboratory parameters. The results of studies conducted to identify the associations of serum complement and autoantibodies with disease activity/severity in SLE are inconsistent and the value of these conventional tests as markers of SLE disease activity is being revisited. A number of potential biomarkers for SLE disease activity has recently emerged.

$CD27_{high}$ plasma cells. In adult SLE patients with active disease, the B cells homeostasis is altered, with significantly decreased naïve B cells and remarkably increased $CD27_{high}$ plasma cells[230]. In pediatric SLE patients, especially those with active disease, a subset of B cells resembling plasma cell precursors has been detected in the peripheral circulation[231]. These observations support a role for autoantibody-producing plasma cells in the pathogenesis of SLE. Jacobi and colleagues reported that the number and frequency of $CD27_{high}$ plasma cells were significantly correlated with disease activity scored by SLEDAI, ECLAM, and the titer of anti-dsDNA antibodies[232]. They also observed that the expansion of the $CD27_{high}$ plasma cell population increased with duration of disease and decreased after effective treatment with immunosuppressive agents[233]. The percentage of $CD27_{high}$ plasma cells has also been suggested as a biomarker to distinguish lupus flare from infection[234].

Cell-bound complement activation products (CBCAPs). Increased levels of cell-bound C4d have been found on reticulocytes (R-C4d), the youngest and short-lived erythrocytes, in lupus patients. The R-C4d levels fluctuate and correlate with clinical disease activity as measured by SLEDAI and SLAM, which supports the potential of R-C4d as a biomarker for monitoring lupus disease activity[235]. In the largest longitudinal lupus biomarker study to date over a 5-year period, after a multivariate analysis, EC4d was shown to be the only biomarker significantly associated with SLE activity measures, after adjusting for serum C3, C4, and anti-dsDNA[236 237].

IFNα and IFN-inducible gene profiles. The type I IFN system family consists of 13 subtypes of IFN-α encoded by 13 genes and a single gene for each of the other family members that include IFN-β, IFN-δ, IFN-ξ, IFN-ω, IFN-κ, IFNε and IFNτ. All type I IFN family members bind to the same IFN-α/β receptor. IFNα and related genes have a prominent role as candidate soluble and genetic biomarkers for lupus disease activity. A subset of SLE patients is characterized by a pattern of up-regulated IFN-inducible genes (termed ''IFN signature'') in peripheral blood mononuclear cells (PBMC), which predicts more severe disease, such as cerebritis, nephritis, and hematologic involvement[238]. Associations of IFN-inducible genes and/or chemokines with increased disease activity, hypocomplementemia, and the presence of specific autoantibodies have been reported in both adult and pediatric SLE patients[193]. The levels of some chemokines, such as CXCL10 (IP-10), CCL2 (MCP-1) and CCL19 (MIP-3B), in serum of SLE patients performed better than traditional laboratory tests and showed strong correlation with disease activity, rising at flare and decreasing with remission[239]. The expression levels of selected IFNα-inducible genes are significantly elevated in concomitance with increased clinical disease activity, active renal disease, decreased C3 levels, positive anti-dsDNA antibodies and anti-RNA-binding protein at a single time point, but not when followed over time[240]. The IFN-inducible gene profile of peripheral blood cells may predict future disease activity in SLE patients. Reeves and colleagues have suggested that expression of CD64 on circulating monocytes is IFN-I inducible and may, therefore, be a surrogate marker for the IFN signature[241].

Binding of immune complexes containing DNA, RNA, or RNA-binding proteins to pDC triggers overproduction of IFNα in SLE patients. High levels of IFNα and IFN-inducible chemokines may in turn lead to activation of autoreactive lymphocytes, dysfunction of regulatory T cells, and dysregulation of endothelial cells and vasculogenesis[242]. The promising data on the IFN signature/ IFN-inducible proteins as biomarkers for SLE disease activity await further investigation in large scale trials.

B lymphocyte stimulator (BLyS) or B-cell activating factor (BAFF). Cross-sectional and longitudinal studies showed that significantly elevated circulating levels of BLyS are found in some SLE patients, correlated with increased anti-dsDNA levels, as well as with current and upcoming disease activity. Changes in serum BLyS levels did not correlate with changes in disease activity and/or specific organ involvement in individual patients[243-247]. SLE patients have significantly higher serum BLyS levels than RA patients[248].

Most recently, James and colleagues reported that BLyS levels were associated with increased lupus disease activity in white but not in African American patients who had higher BLyS levels regardless of disease activity. A significant correlation between BLys levels and serum IFNα activity was demonstrated[249].

Several other cross-sectional studies have identified a growing list of potential biomarkers for monitoring lupus disease activity including both humoral and cell surface molecules, but the data are not always sufficient and consistent, yet, and further confirmatory investigations should be performed.

Biomarkers for specific organ involvement

Around half of patients affected by SLE develop renal involvement, with higher risk for potentially life-threatening complications. Effective methods of detecting lupus nephritis (LN) would improve the quality of life and the prognosis of these patients. Currently, the gold standard for diagnosing and classifying LN is the renal biopsy. Non-invasive conventional markers of disease activity, such as serial measurements of serum creatinine concentrations, proteinuria, complement levels, anti-double-stranded DNA and other antibodies fail to adequately predict renal lupus flares. The evaluation by light microscopy of presence or absence of urinary sediment (deposit of red cells, white cells or urinary casts after centrifugation of the collected urine sample) is often used as a measure of disease activity. The presence of >5 cells per high power field (HPF) in the absence of an infective organism is generally considered as 'active' sediment.

Biomarkers for renal involvement. Besides traditional SLE autoantibodies, the most reliable biomarkers for renal involvement are C4d, NGAL, MCP-1 and TWEAK.

Both cross-sectional and longitudinal studies have indicated that urinary levels of MCP-1 **(uMCP-1)** protein and mRNA are promising biomarker candidates due to specificity for renal activity, sensitivity in predicting renal flares, and demonstrating the capacity to reflect both the severity of flares and the proliferative nature of the histology[250 251].

Neutrophil gelatinase-associated lipocalin **(NGAL)** is a candidate biomarker of lupus nephritis in adult patients. Both serum and urinary NGAL in pediatric patients demonstrated the capacity to predict exacerbation of renal disease and flares[252 253].

Urinary levels of tumor necrosis-like inducer of apoptosis **(uTWEAK)** are significantly higher in lupus patients with active nephritis as compared to those with inactive or no nephritis[251 254].

Composite panels of non-invasive investigations have shown high correlation with the outcome of renal biopsy[255]

Biomarkers for central nervous system involvement. Anti-NR2 antibodies are reported to be detected the circulation of approximately 30% of SLE patients, but their relationship with NP-SLE is controversial[256-259]. Measurement of anti-NR2 antibodies in CSF may be more useful for the diagnosis of NP-SLE than measurement of these antibodies in serum.

A subset of the anti-dsDNA antibodies cross-reacts with a sequence present in the extracellular domain of the NR2a and NR2b subunits of the NMDA receptor and is present in the sera, CSF, and brains of SLE patients with progressive decline in cognitive performance[260].

Other biomarkers for organ involvement. Platelet C4d (PC4d) is associated with occurrence of acute ischemic stroke. In addition, PC4d at baseline is associated with all-cause mortality[261].

Dysfunctional pro-inflammatory HDL (piHDL) and leptin greatly increases the risk of developing subclinical atherosclerosis in patients with lupus[262-264].

Biomarkers studied in the present research project

Whether SLE theoretically represents one disease entity, or is represented by a continuous overlap of etiologically unrelated organ manifestations is far from being established. The term "SLE" may therefore represent a common denominator for a wide variety of intrinsically unrelated disease manifestations.

This is particularly challenging when attempting to determine biomarkers for SLE, including antibodies to dsDNA.

Anti-dsDNA antibodies

As previously mentioned, anti-ds-DNA antibodies are in the vast majority of publications in the last decades referred to as the hallmark of SLE. Anti-dsDNA antibodies are included in all the SLE classification criteria[60-63] over the years and their assessment is useful (but not central) in establishing the diagnosis of SLE.

If I would ask the readers with some medical knowledge to name the first disease anyone recalls when anti-dsDNA antibodies are mentioned, the majority would probably answer "SLE".

It is known that anti-dsDNA antibodies are present in 60–70% of SLE patients and in less than 0.5% of the controls. The presence of anti-dsDNA antibodies correlates with disease activity and increasing titer usually predicts increased disease activity in up to 80% of patients. The majority of SLE patients are found to have anti-dsDNA antibodies at some time during their illness. The presence of anti-dsDNA has been found to precede the onset of lupus symptoms by up to 5 years[265].

In absolute terms, the ability of these antibodies to predict flares is controversial, but in many patients increased levels of anti-dsDNA antibodies may be detected some days or weeks before activation of disease, often with exacerbations of glomerulonephritis[266 267].

The long story of anti-dsDNA started in 1957, when Robbins and colleagues[268] in New York, Ceppellini and colleagues[269] in Milan and Seligmann[4] in Paris described "a serum factor from systemic lupus erythematosus reacting with desoxypentose nucleic acid". Franco Celada[270] was "the slender thread that linked the three discoveries: the youngest and least experienced of all the researchers involved". In his description of the reaction between SLE serum and nuclear material, he writes as follows: "I tried a ring test: layering a DNA solution on a series of dilutions of the serum. The white precipitation at the interface looked like a slow-motion explosion in the side-illuminated tubes. Over the following days I tried all variations, and immediately realized that all DNAs were precipitated, including calf, dog and bacterial DNAs". Friou and colleagues[271] in June, the same year, presented at the ninth international congress of rheumatic diseases in Toronto (Canada) "a globulin factor with a marked affinity for nuclei" and "that the component of nuclei involved was in the nucleoprotein fraction"[272]. Holborow and colleagues[273] supported this finding a couple of months later.

It was obvious since the beginning that anti-DNA antibodies constitute a subset of antinuclear antibodies (which already were known since the beginning of 1950´s). During the following years, further investigations showed that they can be IgM, IgA or any of the subclasses of IgG antibodies, binding single-stranded (ss) DNA, dsDNA, or both.

Antibodies anti-ssDNA can bind any of the multiple DNA components exposed in single strands. In contrast, anti-ds DNA antibodies bind to the ribose–phosphate backbone, base pairs, or particular conformations (right-handed form called B DNA and left-handed form called Z DNA) of the double helix. Some patients with systemic lupus erythematosus have antibodies against both forms, whereas others have antibodies that react preferentially with Z DNA. Studies with monoclonal antibodies have shown that most anti-dsDNA antibodies bind both double- and single-stranded DNA[274].

Healthy subjects may have IgM anti-ssDNA in their repertoire of natural autoantibodies, with low affinity for DNA. IgG antibodies against dsDNA include high-affinity subgroups and are rare in healthy subjects.

Swaak and Smeenk[275] published in 1985 evidence that anti-ds-DNA positive patients without SLE run high risk to develop the disease within a few months. This finding was recently supported by Eriksson et al.[276] who could detect autoantibodies against nuclear antigens, above all anti-dsDNA antibodies, more than 3 years before the onset of SLE and over 6 years before the diagnosis was made.

Pathogenic role of anti-dsDNA.

As we understand the disease today and as mentioned before in this thesis, B cell and T cell autoimmunity to nucleosomes and, in particular, to the individual components of nucleosomes, herein dsDNA and histones, are important in establishing a diagnosis[274 277]. This is further underscored by the fact that anti-chromatin antibodies have the potential to induce nephritis in SLE[278-282]. The pathogenic role of anti-dsDNA antibodies is largely unknown[277 283]. According to current knowledge, antibodies to dsDNA are directly involved in pathogenesis of lupus nephritis[284], lupus dermatitis[285 286] and possibly also in certain aspects of cerebral lupus[287]. How anti-dsDNA antibodies relate to the remaining clinical components listed in current classification criteria[61 63] remains to be determined.

When emphasizing anti-dsDNA antibodies as a central biomarker in SLE, it is important to perceive that these antibodies basically are not representing a homogenous antibody population[288-291]. Growing insight into the factual genesis of anti-dsDNA antibodies challenges the notion of a specific relationship between these antibodies and SLE. For example, antibodies that bind dsDNA may be produced in context of several quite different mechanisms, such as infection-related hapten-carrier systems, molecular mimicry, single gene defects or mutations, as well as the stimulatory effect of apoptotic and secondary necrotic cell debris on the immune system[292-296]. In some cases the stimulus is transient with poor affinity maturation of the antibodies. In other cases the stimulus is sustained allowing maturation of high affinity potentially pathogenic antibodies. These multiple and diverse mechanisms accounting for production of anti-dsDNA antibodies lessen the probability of a specific association of anti-dsDNA antibodies per se with SLE. From the simple statement that individuals in fact may produce anti-dsDNA antibodies without having organ manifestations, like nephritis, indicates the existence of a selection principle that determines the pathogenicity of these antibodies.

Therefore, aside from the problems linked to processes that impose anti-dsDNA antibody production, it is also a question whether, and how, these antibodies are pathogenic. One possibility is that only those antibodies that bind inherently expressed glomerular antigens are pathogenic[297-301]. Alternatively, anti-dsDNA antibodies are pathogenic only when chromatin fragments are exposed in glomeruli[302-307]. This obviously requires that chromatin structures must be retained and exposed in the kidney.

Recently, it has been indicated that anti-dsDNA antibodies are non-pathogenic in absence of exposed chromatin, and that exposed chromatin represents a structural epiphenomenon in absence of antibodies to dsDNA[284 308].

What is the mechanism that accounts for glomerular exposure of chromatin that can be targeted by anti-dsDNA antibodies?

The question is partly answered by recent studies, in which the role of deficient nucleases in the generation of anti-dsDNA antibodies has been discussed[309 310]. It has been demonstrated that an acquired silencing of the renal DNase I enzyme results in impaired chromatin degradation and a consequent retention in the glomerular tissue[284 311 312].

Tissue damage is strongly associated to subsets of anti-dsDNA antibodies, in particular IgG with high-affinity, more than low affinity IgG or IgM anti-dsDNA antibodies. The production of such pathogenic high-affinity molecules occurs in an antigen-driven fashion, a process facilitated by T cells. Thus, B-lymphocytes, which are co-stimulated by both T cells and antigen, undergo continuous selective pressure generating a population of B cells that display and secrete high-affinity immunoglobulin for the stimulating antigen.

Indirectly T cells also produce cytokines, such as TNF-α, IFN-γ, and IL-10, that stimulate B cell division, facilitate immunoglobulin class switching, and promote production of more high-affinity autoantibodies that have been implicated in the tissue damage observed in lupus.

The pathogenicity of anti-DNA antibodies may depend on their complement-fixing capability, their affinity for DNA and cross-reactive antigens, the charge of the antibody molecule or of the immune complex containing it, and the amino acid sequences of associated proteins[274].

The origin of anti-dsDNA antibodies can be disparate. In people genetically predisposed to SLE, some natural anti-dsDNA antibodies can undergo an isotype switch (from IgM to IgG) or somatic gene mutations may result in the production of pathogenic high-affinity IgG antibodies to DNA. Multiple exposures to bacterial, viral, or chemical antigens can lead to the formation of anti-dsDNA antibodies. A part of the production of anti-dsDNA antibodies is induced by self-antigens (particularly nucleic acid–protein complexes) generated as consequence of impaired cell death mechanisms.

Detection of anti-dsDNA antibodies.

During the past years, a wide variety of methods have been used to detect anti-dsDNA antibodies, by measuring secondary events after the formation of immune complexes, such as complement fixation[268 313], precipitation[4 269 314-316], passive hemagglutination[317], bentonite flocculation test[318 319] and fluorescent spot test[320].After the successive introduction of new reliable assays, the aforementioned methods became obsolete. They will not be further discussed in this thesis.

The most widely used and documented methods for the assessment of anti-dsDNA antibodies are radioimmune assay, Crithidia Luciliae immunofluorescence test and ELISA. There are large differences in terms of the sensitivity and specificity of these tests[321-324], most notably among the commercial variants of anti-dsDNA ELISA. In cases of elevated anti-dsDNA titers, it is clinically relevant to exclude other causes, such as infection with Epstein-Barr virus or hepatitis B virus as well as the use of drugs that may cause SLE[322 325].

Radioimmune assay (RIA).

In 1958, Farr[326] described an ammonium sulfate precipitation technique that was applied, around 10 years later, for the first time for the detection of anti-DNA antibodies[327]. The principle is that DNA is soluble in 50% saturated ammonium sulfate, whereas immunoglobulins and immunoglobulin-bound DNA are insoluble. When ammonium sulfate is added to a mixture of radioactive DNA and serum, the precipitate contains radioactivity if DNA is bound to immunoglobulins. The innovation of the Farr assay was the possibility to measure the primary event of the interaction between native DNA and its antibody, regardless of the capacity of immune complexes to determine complement fixation, precipitation or agglutination. Pincus and colleagues[328] reported abnormal binding activity in serum of all the patients with positive and in two thirds of those with negative complement fixation tests for anti-DNA antibodies. The highest binding values were seen chiefly in patients with active SLE renal disease and marked reductions accompanied clinical improvement. The DNA employed must be between 10^5 and 10^7 kDa, double-stranded, with antigenic sites distributed along the DNA molecule[329]. The Farr assay detects primarily high affinity antibodies to dsDNA, regardless of isotypes IgG or IgM and is not exempt from false positive results. Despite the risk of missing SLE with anti dsDNA of low affinity, the Farr assay is, almost 50 years later now, still the gold standard procedure for measuring anti-dsDNA antibodies. Nonetheless, its use in clinical praxis is very rare. The method is time consuming, requires in fact the use of costly labeled native DNA as substrate and the need of a special set up in laboratory[330].

Crithidia Luciliae Immunofluorescence Test (CLIFT).

In 1975, Aarden and colleagues introduced the use of the kinetoplast of Crithidia Luciliae, only containing dsDNA, as substrate for the immunofluorescence (IF) test[331]. CLIFT has high specificity for anti-ds-DNA antibodies with moderate to high avidity and allows isotype testing. It has already been reported that CLIFT is less sensitive than Farr assay[332], probably due to presence of anti-ds-DNA antibodies with different avidity, but the two methods are considered equivalent in clinical practice. The kinetoplast DNA has one of the greatest known degrees of stable curvature[333 334]. Thus, the assays may disclose antibody binding to DNA

structures that are only formed by strong deformations from the more common linear B helical DNA structure. Therefore, antibodies recognizing the kinetoplast DNA of the hemoflagellate Crithidia Luciliae may specifically bind unique structures shared by nucleosomes[333] [334]. This stringent antibody specificity may well reflect structures on eukaryotic nucleosomal DNA that is believed to induce such immune responses in vivo.

Enzyme-linked immunosorbent assay (ELISA)

A large number of different commercial kits and in-house ELISA are currently in use worldwide. ELISA is becoming the prevalent methods used in routine laboratory practice, being a rapid, relatively cheap and sensitive assay.

The available ELISAs can detect high and low affinity anti-dsDNA antibodies, mainly IgG isotype, but potentially also IgM or IgA isotypes. Analysing the relationship between IgG, IgA and IgM anti-dsDNA antibody isotypes and clinical manifestation, a significant association of the IgM isotype with cutaneous involvement and of the IgG isotype with lupus nephritis was found. Moreover, the IgG/IgM ratio of anti-dsDNA antibodies could distinguish patients with lupus nephritis from those without renal involvement[335]. Nonetheless, the heterogeneity of the substrates used, the possible contamination by antibodies anti-ssDNA, other technical problems related to the materials used in the procedures and the lack of standardization of the methods are the most common causes of discordant and false positive results.

The ELISA kits that make use of biotinylated DNA and streptavidin[336] allows a better native structure of antigen and reduces conformational modifications. To minimize nonspecific reactions and to potentially mimic the type of dsDNA presentation in vivo, a nucleosome-complexed ELISA (Anti-dsDNA-NcX ELISA) has been proposed, making use of the strong adhesivity of nucleosomes to attach dsDNA to the solid phase. This ELISA performed very well when it was compared to Farr assay, CLIFT and other conventional ELISA kits[325].

Lupus Erythematosus cell phenomenon

The first laboratory test proposed as a diagnostic tool for SLE was the so called Lupus Erythematosus (LE) cell phenomenon, described in 1948 as a specific finding in bone marrow leukocytes in patients affected by SLE[337].

LE cells are not usually found in peripheral blood, although Sundberg and Lick observed in 1949 that the LE cell phenomenon could form in the buffy coat of peripheral blood after a period of incubation. LE cells have also been found in synovial fluid, cerebrospinal fluid and pericardial and pleural effusions from patients with SLE[338 339].

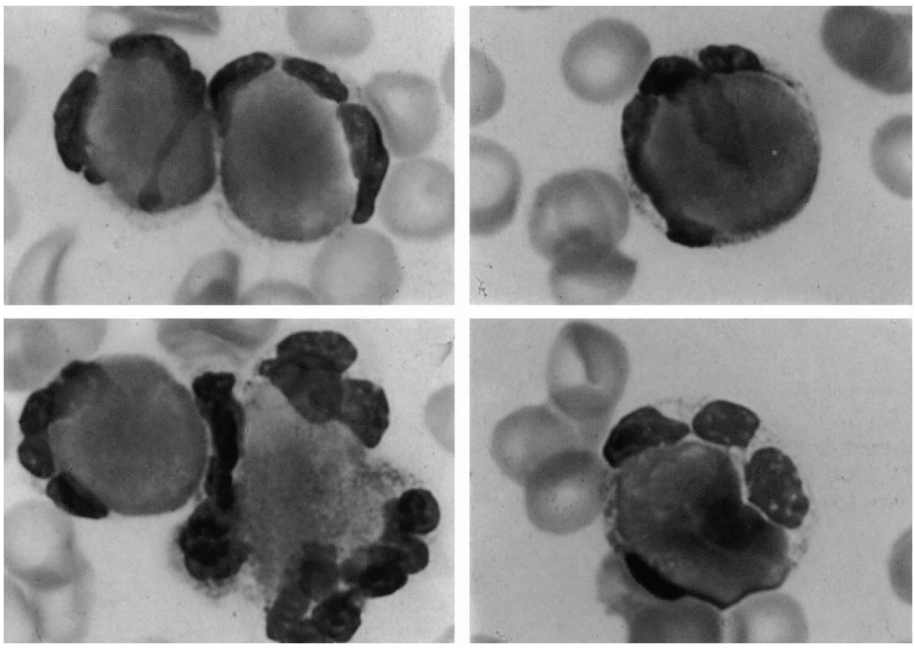
In 1949, Haserick and Bortz showed that the addition of plasma from patients with SLE to bone marrow preparations from normal subjects induced the LE cell phenomenon in these marrows, with the formation of clumps of polymorphs around amorphous masses of nuclear material. The highest number of LE cells developed when plasma from the sickest patient was used. Furthermore, plasma from a patient with discoid lupus failed to induce the phenomenon. Thus, the formation of LE cells appeared to be secondary to a factor in the plasma of patients with SLE[340]. For a while, the LE cell phenomenon was the most specific test available for the diagnosis of SLE, and it supported the autoimmune theory for its pathogenesis. Further studies discovered the ability of the LE factor to bind to nuclei and ribonucleoprotein. We now know that the autoantibodies that lead to the LE cell phenomenon bind histones, in particular H1[341], and dsDNA. In addition, it is shown that LE cells consist of mature polymorphonuclear leukocytes (PMNs), in which the nucleus has been dislocated to the periphery of the cell after engulfment of antibody- and complement-opsonized nuclear material[342 343].

The presence of LE cells has been included in the classification criteria for SLE[60 61] and related to more severe clinical manifestations. The complex assessment of LE cells by light microscopy has been abandoned as a routine test in favor of other diagnostic tools, including anti-dsDNA antibodies analysis.

Phagocytosis of necrotic cell material by polymorphonuclear cells

In 2004, a flow cytometry-based assay was developed as an *in vitro* assessment and quantification of LE cells in patients with suspected SLE[344]. The assay determines the amount of PMNs that perform phagocytosis of necrotic cells (PNC). Its use in clinical practice has not been validated, yet.

In a previous study[345], our group made use of the same method, with minor modifications. We could demonstrate that the outcome of the test is often positive in SLE patients with increased disease activity. Moreover, the phagocytosis seems to be associated with oxidative burst activity, it is mediated by FcγRIIA, FcγRIIIB and CR1 in combination, it occurs in presence of high levels of different anti-histone antibodies and it is more efficient when the classical pathway of the complement system is functional and active.



The LE cell. The large homogeneous areas adjacent to these polymorphs' nuclei each contain the nucleus of another being digested. Free lysed nuclear material can also be seen in the lower left example (reproduced with permission, courtesy Dr G. A. McDonald).

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S100A8/A9

S100A8/A9, or calprotectin or MRP8/14, is a heterodimer consisting of one S100A8 molecule (also known as calgranulin A or myeloid-related protein – MRP – 8 or p8) and one S100A9 molecule (calgranulin B or MRP 14 or p14). The binding of the two components requires the presence of Zn^{2+} and/or Ca^{2+}.

S100A8 and S100A9 are constitutively expressed in neutrophils, monocytes, and dendritic cells, but can be induced upon activation in other cell types such as mature macrophages, vascular endothelial cells, fibroblasts and keratinocytes. In neutrophils, S100A8 and S100A9 constitute around 50% of all cytosolic proteins, compared to only about 1% in monocytes.

The protein S100A8/A9 was first described in 1980 as a marker of turnover of leukocytes and was referred to as L1[346]. Increased levels of a serum protein were reported 2 years later in children with cystic fibrosis (CF) and it was named CF antigen[347]. In 1985, the gene of CF antigen was mapped in chromosome 1[348]. Two years later, two proteins of macrophage expression were described in patients affected by RA. Being their molecular weight 8 and 14 kDa, they were named MRP8 and MRP14, respectively[349]. Only in 1988, it was discovered that L1, CF antigen and MRP8/14 were the same protein and the name L1 was suggested[350], waiting for a better name that could recall the features of the protein. In 1990, the term calprotectin was used for the first time, to emphasize the antimicrobial (protectin) activity of this calcium (cal) binding protein[351]. It was later found that the two components of the heterodimeric protein belong to a superfamily of proteins having in common the solubility in 100% ammonium sulfate solution, the so-called S100 proteins[352].

S100 proteins. S100 proteins in humans are, according to the most updated nomenclature[353] and review[354], 21 small acidic proteins composed of 2 EF-hand regions connected by a central hinge region. S100 proteins are the largest of the EF-hand calcium binding proteins. The N-terminal EF-hand has a 14 amino acid consensus sequence motif (flanked by 2 helices, H-I and H-II) and is called the 'S100-specific' or 'pseudo' EF-hand. The EF-hand at the C-terminus contains a classical $Ca2^{+}$-binding motif, common to all EF-hand proteins, with a typical sequence signature of 12 amino acids (flanked by 2 helices, H-III and H-IV). Upon Ca^{2+}-binding, S100 proteins undergo a conformational change, with reorientation of helix H-III, which opens the structure and exposes a wide hydrophobic surface, functioning as interaction site of S100 proteins with their target proteins.

Seventeen of human S100 genes, herein S100A8 and S100A9, are tightly clustered in the chromosome region 1q21. The S100 genes structure often contains three (the first non-coding) exons and two introns.

S100 proteins are involved in a variety of cellular processes such as cell cycle regulation, cell growth, cell differentiation or motility. S100 proteins form homo- and heterodimers, as well as oligomers with functional diversity.

S100A9 is different from other S100 proteins because of its long C-terminus, which is extremely flexible. S100A8 and S100A9 tend to form homo- and heterodimers in absence of Ca^{2+} and associate to higher-order oligomers in a Ca^{2+}-dependent manner. The formation of tetramers may also be triggered by zinc[355-357].

Pathogenic role of S100A8/A9.

Extracellular S100A8/A9 is primarily released from activated or necrotic neutrophils and monocytes/macrophages and is involved in the pathogenesis of various diseases with an inflammatory component.

The calgranulins belong to the damage-associated molecular pattern molecules (DAMPs), cell or tissue components released upon injury, which modulate inflammatory reactions by interacting with pattern recognition receptors (PRR), such as the receptor for advanced glycation end products (RAGE) and TLR4[358].

The RAGE is a member of the immunoglobulin-like receptor superfamily and is expressed on the surface of different cells, such as vascular smooth muscle cells, mononuclear phagocytes and endothelial cells.

S100A8/A9 binding to RAGE leads to MAP kinase phosphorylation and NF-kB activation, promoting leukocyte production. RAGE activation leads to further enhancement of S100A8/A9 production, creating a putative positive feedback loop in chronic inflammation[358].

S100A8/A9 binding triggers MyD88-mediated TLR4 signalling, leading to NF-kB activation and secretion of pro-inflammatory cytokines such as TNFα and IL-17.

The S100A8/A9-TLR4 interaction has been shown to be involved in the pathogenesis of systemic infections, autoimmune diseases, malignancy, and acute coronary syndrome[359].

Concentration-dependent anti-inflammatory functions of S100A8, S100A9, and S100A8/A9 have been reported, such as inhibition of ROS production[360].

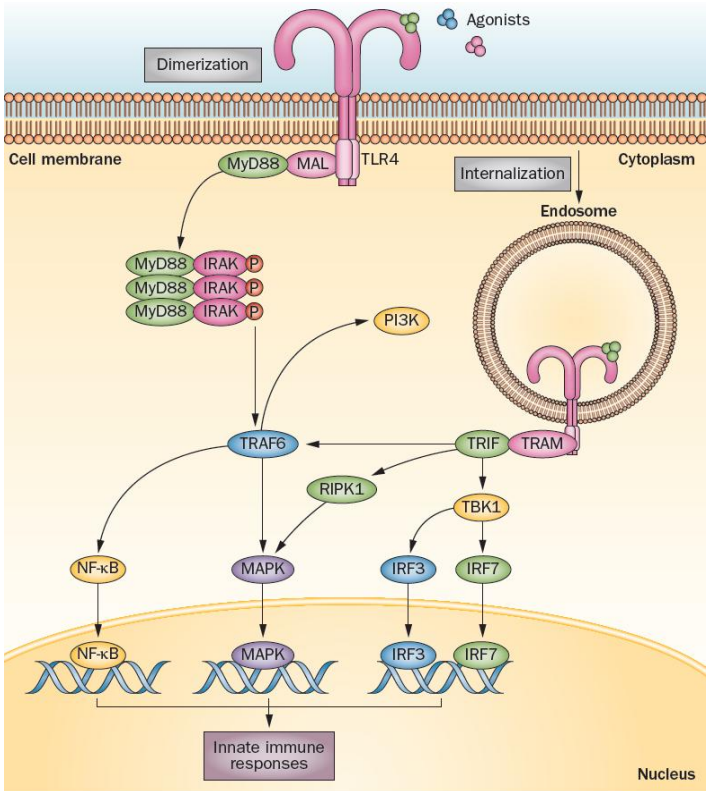
Increased serum levels of S100A8/A9 have been described as a predictor of cardiovascular events and also as a marker to distinguish between acute coronary syndrome and stable coronary disease, but the data are inconsistent[361-363].

S100A8/A9 proteins are mostly involved in inflammatory diseases and their expression is low in healthy people. S100A8 and S100A9 are associated with chronic inflammatory diseases, including bowel diseases and chronic periodontitis,

and both proteins are involved in wound repair by re-organization of the keratin cytoskeleton in the injured epidermis.

Serum S100A8/A9 levels are increased in several inflammatory diseases, including SLE. In SLE patients, serum concentrations of S100A8/A9 correlate with disease activity, indicating that these proteins could be involved in the pathogenesis of the disease[359 364]. Our research group documented that cell surface S100A8/A9 is detectable on all leukocyte subpopulations except for T cells and it is enhanced on pDC cell surface in SLE patients with active disease, especially upon immune complex stimulation. Plasmacytoid DCs, monocytes and PMNs can synthesize S100A8/A9[365].

We could also demonstrate that serum levels of S100A8/A9 were elevated in inactive SLE patients as compared with healthy individuals. Moreover, increased S100A8/A9 and S100A12 in serum of SLE patients are associated with a history of CVD and presence of organ damage[366].



TLR4 signalling. Upon ligand-induced TLR4 dimerization, the MyD88-dependent signalling pathway is activated. TLR4 can also internalize into endosomes and signal by the MyD88-independent pathway. TLR4 signalling activates multiple transcription factors, including MAPK, interferon regulatory factors and NF-κB,which promote innate immune responses, including the induction of iNOS and cyclooxygenase 2. Abbreviations: IRAK, IL-1 receptor associated kinase; IRF3, interferon-regulatory factor 3; IRF7, interferon-regulatory factor 7; MAL, MyD88 adaptor-like; MAPK, mitogen-activated protein kinase; MyD88, myeloid differentiation primary response gene 88; NF-κB, nuclear factor κB; iNOS, inducible nitric oxide synthase;P, phosphate; PI3K, phosphatidylinositol-4,5-bisphosphate 3-kinase; RIPK1, receptor-interacting serine/threonine-protein kinase 1; TBK1, TANK-binding kinase 1; TLR4,Toll-like receptor 4; TRAF6, TNF receptor associated factor 6; TRAM, TRIF-related adapter molecule; TRIF, TIR-domain-containing adapter inducing interferon-β.

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Osteopontin

Osteopontin (OPN), also known as early T lymphocyte activation-1 or secreted phosphoprotein 1, is a member of the small integrin-binding ligand N-linked glycoprotein (SIBLING) family proteins. Human OPN gene is mapped on the long arm of chromosome 4 (4q21–4q25) and its expression is affected by cytokines, like IL-1β, IL-6, TNF-α, IFN-γ, vitamin D and hormones, such as estrogen, angiotensin II and glucocorticoids. Osteopontin function is highly modified by post-translational modifications, including phosphorylation, O-linked glycosylation, sialysation and tyrosine sulfation.

OPN interacts with cells via two binding domains, the adhesive RGD motif (arginine–glycine–aspartate domain) and the SVVYGLR domain. OPN contains an aspartate-rich region near the C-terminal sequence, which is exposed, following proteolysis by thrombin, and is able to interact with the CD44 receptors.

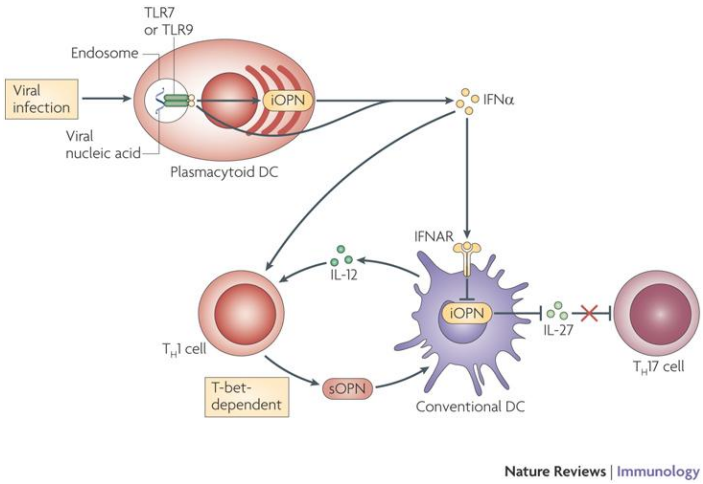
OPN is a pleiotropic protein found in many organs and body fluids. It was initially identified in 1986 as a major extracellular sialoprotein of bone matrix, linking as a bridge (pons in Latin) bone cells and hydroxyapatite, the main inorganic constituent of human bone tissue[368]. Besides osteoblasts and osteocytes, OPN is produced by breast epithelial cells, neurons and various immune cells, such as B-cells, T-cells, natural killer (NK) T cells, NK cells, macrophages, neutrophils and dendritic cells (DCs). Due to the fact that OPN is expressed by many different cell types of the immune system, is up-regulated in response to injury and inflammation and regulates immunological response, it may be classified as a cytokine[369 370].

Osteopontin is highly expressed by macrophages and regulates their migration, activation, capacity for phagocytosis and nitric oxide production [369 370]. It has been demonstrated that OPN is a chemoattractant for neutrophils[370 371] and induces DCs maturation.

Recent findings revealed that the intracellular isoform of OPN enhances IFN-α expression through activation of the IRF7 upon TLR 9 stimulation in pDCs[372]. The absence of OPN in mice impairs IFN-α production by pDCs[372].

OPN promotes activation of T lymphocytes, and regulates the T-helper 1 (T_H1)/T_H2 balance. In addition, some studies suggested that OPN enhances IL-17 producing T_H17 cell responses by inhibiting the production of IL-27 and IL-17 inhibitor produced by pDC[373 374].Moreover, OPN activates and stimulates antibodies production by B lymphocytes[375 376].

Via interaction with αvβ3 integrin, OPN up-regulates IL-12. Moreover, the interaction with OPN may activate the complement factor H (CFH), which leads to disabled formation of MAC. Through CD44 receptor, OPN down-regulates IL-10[377] and may lead to anti-apoptotic signals via Akt-phosphorilation.



The expression of the intracellular isoform of Osteopontin (iOPN) is induced in plasmacytoid dendritic cells (pDCs) following ligation of Toll-like receptor 7 (TLR7) or TLR9, for example, during viral infection, and results in enhanced production of interferon-alpha (IFNalpha). This production of IFN-α by pDCs contributes to T helper 1 (T_H1)-cell responses during viral infection. Activated T cells produce high levels of the secreted isoform of Osteopontin (sOPN), which is under the control of the transcription factor T-bet. This triggers T_H1-cell responses through the induction of secretion of pro-inflammatory cytokines, including interleukin-12 (IL-12), by antigen-presenting cells. iOPN expressed by conventional DCs induces T_H17-cell responses by blocking the expression of IL-27, which suppresses the development of T_H17 cells. Type I IFN receptor (IFNAR) engagement suppresses the expression of iOPN, thereby removing the 'brake' on IL-27 expression and, consequently, on attenuated T_H17-cell responses.

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OPN in SLE.

OPN is considered to be an effective biomarker for a number of cancers and immune-mediated diseases[379]. In 1989, the first report was published to connect OPN to immunity[380] and other contributions followed suggesting that OPN participates in the pathogenesis of some autoimmune diseases[373 381 382]. In 1995, Katagiri and colleagues for the first time evaluated whether elevated OPN level can be detected in patients with autoimmune diseases[383]. They found significantly higher levels in patients than in healthy donors and described two isoforms of OPN: large (64 kDa) and small (32 kDa). The latter is a thrombin-cleaved isoform

derived from the large OPN and exposes an epitope for the integrin receptors α4β1, α9β1 and α9β4[384 385]. Other contributions have later confirmed presence of elevated plasma OPN concentration in SLE[386-389]. In patients with renal involvement, a positive significant correlation was found with SLE disease activity index and with IL-18. The latter is a pro-inflammatory cytokine that can induce IFN-α to promote T_H1 differentiation and exacerbation of disease activity, probably with the synergic contribution of OPN[388]. Similar results have been described in children affected by SLE[386], along with higher titer of anti ds-DNA antibodies, elevated IFN-α and upcoming increased SLEDAI after six months[387]. Therefore, the production of OPN is probably associated with SLE activity and may serve as a potential marker of SLE-related organ damage[387]. Elevated serum levels of OPN significantly correlated also with anemia in SLE and were decreased in patients on treatment with renin-angiontensin system antagonists[389]. OPN was reported to be highly expressed in lupus prone mice, especially by CD4-/CD8- T cells, with significant association with renal damage[390 391]. Miyazaki and colleagues reported that OPN gene polymorphism induces enhanced expression of immunoglobulins (IgG3, IgG2a and IgM) and cytokines (IFN-γ, TNFα, IL-1β) that play important roles in lupus mice models and in human SLE[390]. A number of studies demonstrated that increased plasma concentration, as a result of OPN gene polymorphism and increased protein expression, was associated with SLE susceptibility and/or clinical manifestations of the disease in humans[392]. A total of 13 single nucleotide polymorphisms (SNPs) in OPN gene were identified[393], two of which (rs7687316 and rs9138) were significantly associated with SLE, suggesting that they predispose to high production of OPN and susceptibility to SLE. OPN genetic variants seem to have a key role in creating a background that favors lymphocyte accumulation and development of autoimmunity. In fact, OPN may stimulate proliferation of lymphocytes and simultaneous inhibition of their apoptosis. Alternatively, it may induce Th1 responses and potentiate polyclonal activation of B cells[378]. Significant differences between men and women have been reported concerning the frequencies of genotypes associated with SLE and lupus nephritis, suggesting that OPN gene polymorphism is associated with SLE, especially in males[204 394]. Other studies documented associations of different polymorphisms with clinical features, such as photosensitivity, thrombocytopenia, hemolytic anemia[395]. The mechanism by which OPN gene polymorphism modulates serum IFN-α is unclear but murine data suggest a role of OPN in IFN-α production by pDC[372].

Evidence of the role played by OPN in immune processes stimulates the idea of a possible application in clinical practice. A therapeutic approach that aims at modulating OPN in has been tried in the treatment of cardiovascular and neoplastic diseases with promising outcome[396-398].

The present investigation

Aims

The aim of the studies reported in paper I and II was to investigate, in an unbiased approach, the diagnostic and predictive role played by anti-dsDNA antibodies and to correlate their presence with individual organ involvement or with certain clinical phenotypes, regardless of diagnosis.

In the studies reported in paper III and IV, the aim was to investigate whether novel laboratory tools may be considered as biomarkers in SLE patients and may contribute to a more accurate diagnosis, prediction of clinical features, tailored follow-up and treatment of patients, hence to a better understanding of the pathogenesis of the disease.

Part one: The Scandinavian Anti-DNA study (Papers I and II)

In a realistic clinical scenario, the physician often faces the challenging task to early diagnose diseases in patients with recent onset of rheumatic symptoms. After the evaluation of the clinical features, the use of helpful laboratory tools may contribute to better definition of the diagnosis and management of the disease.

Despite anti-dsDNA antibodies are considered central laboratory tools in SLE and are often used in the diagnostic process of the disease, their accuracy has been mainly investigated in selected patients affected by SLE or other well defined diseases.

The present investigation, called “Scandinavian anti-DNA study” was started in 2003, after approval by the local ethical committees of the participating centres in Tromsø, Norway (project no. P Rek Nord 03/2004), Lund, Sweden [project no. LU 30-03 (LU-P12-03)] and Copenhagen, Denmark [project no. (KF) 01-024/03].

Study design

To address the main questions of this part of the research project, we decided to investigate a population of patients with unknown diagnoses, referred for the first time to a rheumatologist because of recent onset of rheumatic symptoms.

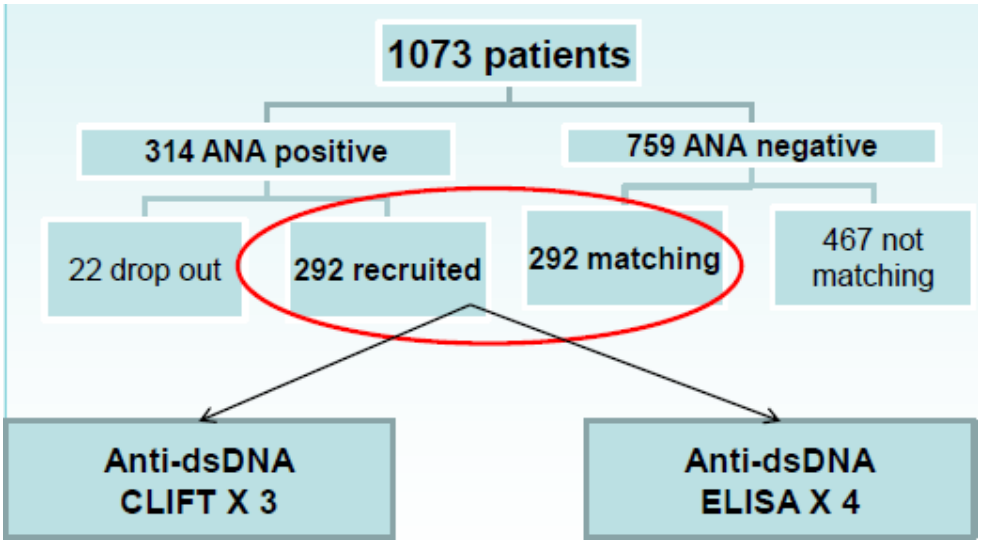
The departments in Tromsø and Lund received referrals for a broad range of rheumatic symptoms, whereas in Copenhagen only tertiary referrals concerning suspected or confirmed inflammatory rheumatic diseases were received.

Exclusion criteria were: age below 15 years, established autoimmune disease, treatment with any biologic drug, corticosteroids (equivalent Prednisolon >20 mg/day), immune-modulator, immune-suppressive or cytostatic drugs. Patients that previously had been examined by any rheumatologist and patients unable to fully collaborate in the study (unconfident with the language, actual impairment of cognition, speech, hearing or memory) were also excluded.

During the first visit at the participating centres, the patients were examined by a rheumatologist, unaware of the result of the anti-dsDNA testing, who made an initial clinical diagnosis, based on the signs, symptoms and results of laboratory tests. No systematic use of classification or diagnostic criteria was done to make diagnosis in the recruited patients. The initial diagnosis was updated and verified after about five years follow-up, at censoring.

The diagnoses were grouped as follows: SLE; other autoimmune connective tissue disease (Sjögren´s syndrome, systemic sclerosis, dermatomyositis, polymyositis, mixed connective tissue disease, undifferentiated connective tissue disease, anti-phospholipid syndrome); systemic inflammatory disease (polymyalgia rheumatica, temporalis arteritis, sarcoidosis, systemic vasculitis, adult onset Still´s disease, fever of unknown origin); inflammatory joint disease (undifferentiated arthritis, rheumatoid arthritis, psoriatic arthritis, ankylosing spondylitis, reactive arthritis, juvenile idiopathic arthritis, gout); non-inflammatory joint disease (osteoarthritis, orthopaedic disorder, congenital musculoskeletal disorder); soft tissue rheumatism (fibromyalgia, tendinitis, entesitis); arthromyalgia (any musculoskeletal pain of unknown origin); dermatological disorder (psoriasis, discoid lupus erythematosus, skin vasculitis and any other isolated skin disease); non-rheumatic disease (any other verified disease without evidence of rheumatic disorder); unspecified (no diagnosis or diagnosis that do not fit in the other groups).

Altogether, 1073 patients were recruited from February 2003 to December 2007. After preliminary ANA screening, all the 292 ANA positive patients were matched with 292 ANA negative patients and further investigated, with detailed definition of the past and current clinical manifestations, as well as with cross-sectional assessment of anti-DNA antibodies, by different methods. Each centre received aliquots of serum from all patients allowing a simultaneous analysis with all anti-DNA antibody assays implemented in this study.



Assessment of biomarkers

In the participating centres, respective current routine methodology was used for detection of ANA. In Copenhagen and Lund screening for ANA was performed by indirect immunofluorescence (IIF) technique. In Copenhagen, HEp-2 cells and patient sera in dilution 1/160 were used together with an FITC-labeled anti-human IgG conjugate. In Lund, Hep-2 or Hep-20-10 cells and serum dilution 1/400 were used. In Tromsø, the detection of ANA was performed with the ELISA Varelisa ReCombi ANA Screen as recommended by the manufacturer.

The 584 patients in the study group had IgG anti-DNA antibodies determined by the various assays available at the participating centres.

IIF test with the hemoflagellate Crithidia Luciliae as substrate (CLIFT) was used by all the centres for detection of anti-dsDNA antibodies, according to the instructions of the manufacturer. The same commercial kit was used in Copenhagen and Tromsø. In paper I, it is referred to as CLIFT A, whereas in paper II it is referred to as CLIFT1 and CLIFT2, respectively. Another commercial kit was used in Lund, referred to as CLIFT B and CLIFT3 in paper I and II, respectively. All the assessments were categorized as negative or positive, based on the fluorescence intensity detected.

In paper II, the additive determination of anti-DNA antibodies by ELISA was performed, using three different solid phase ELISA tests (one in Copenhagen and two in Tromsø), all according to the instructions of the manufacturer. Moreover, one in-house solution phase anti-dsDNA ELISA (SPADE), which measures antibody-binding to dsDNA in solution using dsDNA (pUC18 DNA) biotinylated as recommended by the manufacturer, was used in Tromsø.

Table 1 Overview of anti-dsDNA tests used in the participating laboratories for analysis of serum aliquots from all patients

Name	Methodology	Antigen	Reference interval	Manufacturer	Centre
CLIFT 1	IIF	Crithidia luciliae kinetoplast	<titre 10	ImmunoConcept	CPH
CLIFT 2	IIF	Crithidia luciliae kinetoplast	<titre 10	ImmunoConcept	Tromsø
CLIFT 3	IIF	Crithidia luciliae kinetoplast	<titre 10	Euroimmune	Lund
EliA	Solid phase ELISA	Recombinant plasmid dsDNA	<10 IU/mL	Phadia	Tromsø
SPADE	Solution phase ELISA	Biotinylated plasmid dsDNA and biotinylated, S1 nucleased human dsDNA	<1 AU/mL	Inhouse	Tromsø
Varelisa 1	Solid phase ELISA	Recombinant plasmid dsDNA	<55 IU/mL	Phadia	Tromsø
Varelisa 2	Solid phase ELISA	Recombinant plasmid dsDNA	<35 IU/mL	Phadia	CPH

AU, arbitrary units; CLIFT, Crithidia Lucillia Immunofluorescence Test; CPH, Copenhagen; IIF: indirect immunofluorescence technique; IU, international units; SPADE, solution phase anti-dsDNA ELISA.

From: M Compagno, OP Rekvig, AA Bengtsson et al, Clinical phenotype associations with various types of anti-dsDNA antibodies in patients with recent onset of rheumatic symptoms. Results from a multicentre observational study. Lupus Sci Med 2014;1:e000007[399]

Clinical phenotype description

In paper II, the association between outcome of anti-DNA antibodies measurements and certain clinical manifestations was studied.

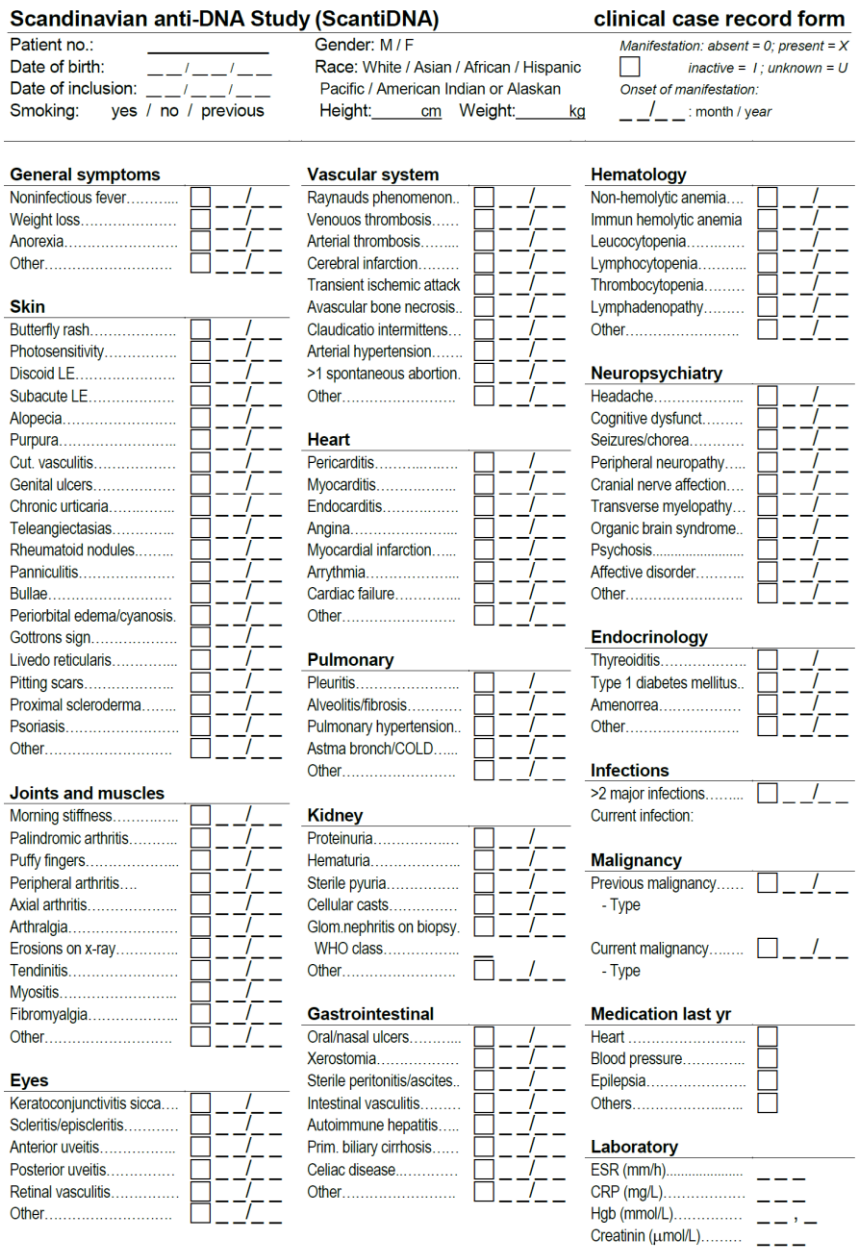
A systematic clinical data chart (page 88), including also relevant routine laboratory parameters, was completed for all patients ensuring a common trunk of data on which the clinical phenotypes of the patients could be characterized. Consensus across the participating centres as to the content of the clinical data set was obtained through a Delphi-like process, spanning over four meetings, during which a gross list of manifestations was reduced to a feasible data set. The gross list consisted of a wide range of clinical manifestations suggested by the study participants, including current, previously used or proposed items from various classification systems of autoimmune connective tissue diseases. A uniform assessment of the final clinical data set was assured by agreement upon the definition of the various manifestations achieved prior to the study (pages 89-94). The clinical features were recorded as being absent ever, ongoing/active, previous/inactive or unknown and the date any manifestations first appeared was noted. In the present study, calculations were based on the presence ever of a manifestation.

Statistical analysis

After finalizing data retrieval, all demographic data, laboratory outcomes, diagnoses and clinical manifestations from all the recruited patients were registered in a database using Microsoft® Office Access software and descriptive statistical analyses were performed using IBM® statistics software SPSS.

In addition, in paper I, sensitivity (Se), specificity (Sp), positive predictive value (PPV), likelihood ratio of positive (LR+) and negative (LR-) result for the diagnosis SLE were determined for the evaluation of the diagnostic accuracy of ANA and each CLIFT assessment. Moreover, the agreement of results of the performed assays was calculated by kappa statistics.

In paper II, the association between presence of anti-DNA antibodies and clinical features was analyzed by performing binary univariate and multivariate logistic regression analysis, being dichotomized anti-dsDNA results as dependent variable and each clinical manifestation registered in the database as dichotomized explanatory variables. Principal component analysis (PCA) was also performed, using dichotomized data, to search for clusters of clinical features and outcome of antibody assessments, which display the highest degree of co-variation and influence in discriminating the patients.



Scandinavian anti-DNA Study (ScantiDNA) — **clinical case record form**

Patient no.: _____
Date of birth: __ __ / __ __ / __ __
Date of inclusion: __ __ / __ __ / __ __
Smoking: yes / no / previous

Gender: M / F
Race: White / Asian / African / Hispanic / Pacific / American Indian or Alaskan
Height: _____ cm Weight: _____ kg

Manifestation: absent = 0; present = X
inactive = I ; unknown = U
Onset of manifestation:
__ __ / __ __ : month / year

General symptoms
Noninfectious fever
Weight loss
Anorexia
Other

Skin
Butterfly rash
Photosensitivity
Discoid LE
Subacute LE
Alopecia
Purpura
Cut. vasculitis
Genital ulcers
Chronic urticaria
Teleangiectasias
Rheumatoid nodules
Panniculitis
Bullae
Periorbital edema/cyanosis
Gottrons sign
Livedo reticularis
Pitting scars
Proximal scleroderma
Psoriasis
Other

Joints and muscles
Morning stiffness
Palindromic arthritis
Puffy fingers
Peripheral arthritis
Axial arthritis
Arthralgia
Erosions on x-ray
Tendinitis
Myositis
Fibromyalgia
Other

Eyes
Keratoconjunctivitis sicca
Scleritis/episcleritis
Anterior uveitis
Posterior uveitis
Retinal vasculitis
Other

Vascular system
Raynauds phenomenon
Venouos thrombosis
Arterial thrombosis
Cerebral infarction
Transient ischemic attack
Avascular bone necrosis
Claudicatio intermittens
Arterial hypertension
>1 spontaneous abortion
Other

Heart
Pericarditis
Myocarditis
Endocarditis
Angina
Myocardial infarction
Arrythmia
Cardiac failure
Other

Pulmonary
Pleuritis
Alveolitis/fibrosis
Pulmonary hypertension
Astma bronch/COLD
Other

Kidney
Proteinuria
Hematuria
Sterile pyuria
Cellular casts
Glom.nephritis on biopsy
WHO class
Other

Gastrointestinal
Oral/nasal ulcers
Xerostomia
Sterile peritonitis/ascites
Intestinal vasculitis
Autoimmune hepatitis
Prim. biliary cirrhosis
Celiac disease
Other

Hematology
Non-hemolytic anemia
Immun hemolytic anemia
Leucocytopenia
Lymphocytopenia
Thrombocytopenia
Lymphadenopathy
Other

Neuropsychiatry
Headache
Cognitive dysfunct
Seizures/chorea
Peripheral neuropathy
Cranial nerve affection
Transverse myelopathy
Organic brain syndrome
Psychosis
Affective disorder
Other

Endocrinology
Thyreoiditis
Type 1 diabetes mellitus
Amenorrea
Other

Infections
>2 major infections
Current infection:

Malignancy
Previous malignancy
- Type

Current malignancy
- Type

Medication last yr
Heart
Blood pressure
Epilepsia
Others

Laboratory
ESR (mm/h) ___
CRP (mg/L) ___
Hgb (mmol/L) __ , __
Creatinin (µmol/L) ___

From: M Compagno, OP Rekvig, AA Bengtsson et al, Clinical phenotype associations with various types of anti-dsDNA antibodies in patients with recent onset of rheumatic symptoms. Results from a multicentre observational study. Lupus Sci Med 2014;1:e000007[399]

ScantiDNA-study nomenclature

Non-infectious fever	The objective determination of an elevation of body temperature above the normal range (i.e. 37 ± 1 °C).
Weight loss	Decrease in body weight that is not voluntary.
Anorexia	Reduction or loss of appetite or desire for food.
Butterfly (Malar) Rash	Diffuse or patchy erythema of the malar eminence(s). Lesions may be flat or raised, involving cheeks and/or the bridge of the nose but tending to spare the nasolabial folds; may be unilateral and may involve adjacent or other areas.
Photosensitivity	An unusaul skin reaction from exposure to sunlight (typically UV-B). Examples would be persistent erythema, edema, urticaria or vesicular-bullous lesions, located in sun-exposed areas.
Discoid LE (Discoid rash)	Rash occurring predominantly (but not exclusively) in sun exposed areas and characterized by erythematous, raised patches with adherent keratotic scaling and follicular plugging; atrophic scarring, telangiectasias, hyperpigmentation (peripheral) and hypopigmentation (central) may be present in older lesions.
Subacute LE	Widespread photosensitive, nonscarring eruptions, either papulosquamos (psoriasiform) or annular.
Alopecia	An abnormal patchy or diffuse loss of hair, particularly scalp hair, non-scarring.
Purpura	Intracutaneous or subcutaneous hemorrhage as evidenced by red to dark purple areas in the skin.
Cutaneous vasculitis	Confirmed by skin biopsy or convincing clinical presentation when present on acral sites where biopsy is not feasible.
Genital ulcers	A break in the skin or mucous membrane found on the penis, scrotum, labia, vestibule or vagina. Lesions may be painful or painless, single or multiple, recurrent or persistent.
Chronic urticaria	A disorder of the superficial skin consisting of well circumscribed discrete wheals with erythematous raised serpiginous borders and blanched centers. It is usually intensely pruritic, and may be localized or generalized.
Teleangiectasias	Visible macular dilatation of superficial cutaneous blood vessels. These blood vessels collaps upon pressure and fill slowly when pressure is released.
Rheumatoid nodules	Firm, usually painless lumps of variable size found in patients with rheumatoid arthritis. Rheumatoid nodules are commonly foun over areas subject to mechanical trauma (e.g., elbows, heels, walls of olecranon bursa), and occasionally in various internal organs such as lungs and heart.
Panniculitis	Nodular, subcutaneous angiitis with fat-cell necrosis or clinically erythema nodosum.

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Bullae	Vesicular elevation of the cuticle containing transparent watery fluid.
Periorbital oedema or cyanosis	Violaceous periorbital erythema often with upper eye lid swelling or periorbital oedema.
Gottron´s sign	Erythematous patches. Scaly hyperemic patches present over the extensor surface of the knuckles (DIP, PIP and MCP). The eruption may have atrophic features as well.
Livedo reticularis	Reddish/cyanotic reticular discoloration of the skin. Appears on legs, arms, adn torso.
Pitting scars	Digital scarring with loss of substance after acral ulcers.
Proximal scleroderma	Thickening, tightening, nonpitting induration of the skin of both extremities proximal to the MCP (or MTP) joints and the trunk (anterior chest, abdomen, upper or lower back or flanks).
Psoriasis	A chronic hyperkeratotic recurrent skin disorder most often characterized by somewhat raised, sharply marginated papules or plaques which are scaling and distributed predominantly on the scalp, elbows, knees, chest, umbilicus, back and buttocks. Frequent involvement of the fingernails and toenails is present.
Morning stiffness	The subjective complaint of localized or generalized lack of easy mobility of the joints upon arising.
Puffy fingers	A diffuse, usually nonpitting increase in soft tissue mass of the digits extending beyond the normal confines of the joint capsule.
Polyarthritis	Symmetric involvement of more than three joints with clinical signs of synovitis.
Oligoarthritis	Clinical signs of synovitis in three or less joints, often asymmetric.
Axial arthritis	Radiographic signs of sacroiliitis or inflammatory spondylarthropathy.
Arthralgia	Subjective reporting of pain in the joints.
Erosions on x-ray	An erosion is a localized area of bone destruction at or near the joint surface.
Tendinitis	Tenosynovitis determined clinically or by ultrasound.
Fibromyalgia	Widespread pain and tender points as defined by ACR.
Myositis	Muscle weakness accompanied by elevated plasma levels of muscle enzymes. The myopathy is further confirmed by muscle biopsy and/or electromyography.
Keratoconjunctivitis sicca	Confirmed by ophthalmological evaluation using Schirmer test, break up time or Rose Bengal dye.
Scleritis or Episcleritis	Clinical signs of inflammation of the sclera and episclera.
Anterior uveitis	Inflammation of the iris (iritis) or of the iris and the ciliary body (iridocyclitis) is referred to as anterior uveitis and results in photophobia, some decrease in visual acuity, and a variable degree of ocular pain. In contrast to the acute anteroir uveitis, the chronic anterior uveitis associated with juvenile rheumatoid arthritis is frequently asymptomatic.

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Posterior uveitis	Inflammation of the choroid usually involves the retina and the term posterior uveitis and chorioretinitis are often used interchangeably.
Retinal vasculitis	Observed by ophthalmoscopy or by a fluorescein. angiogram.
Raynaud´s phenomenon	Sudden, reversible "dead white" pallor of an acral structure (e.g., fingers, whole hand, toes, tip of nose, earlobe or tongue), precipitated by cold exposure or emotion.
Venous or arterial thrombosis	Thrombosis should only be recognized when clinical suspicion is confirmed by relevant paraclinical method.
Cerebral infarction	Confirmed by relevant neuroimaging technique (CT, MRI)
Transient ischemic attack	Clinical picture of cerebral infarction with full remission of symptoms within 24 hours.
Avascular bone necrosis	Confirmed by conventional radiography, CT or MRI.
Claudicatio intermittens	Muscle pain (ache, cramp, numbness or sense of fatigue), classically in the calf muscle, which occurs during exercise and is relieved by a short period of rest.
Arterial hypertension	Blood pressure > 140/90 and/or commencement of antihypertensive treatment.
>1 spontaneous abortion	Note time for 2nd spontaneous abortion.
Endocarditis	Non-infectious endocarditis verified by ultrasonography.
Myocarditis	Myocarditis may cause arrythmias and/or cardiac failure and confirmed by myocardial biopsy.
Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
Angina pectoris	Severe chest pain due cardiac ischemia without signs of myocardial infarction.
Myocardial infarction	Confirmed by elevated cardiac enzyme levels and electrocardiogram.
Arrythmia	Atrial or ventricular arrythmias, conduction disturbances documented by electrocardiogram.
Cardiac failure	Includes both right and left ventricular failure.
Pleuritis	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
Alveolitis / fibrosis	Active inflammatory alveolitis and/or pulmonary fibrosis confirmed by bronchoalveolar lavage, high resolution CT or conventional radiography.
Pulmonary hypertension	Mean pulmonary artery pressure exceeding 15 mmHg calculated by means of echocardiography or measured by cardiac catheterization.
Asthma or COLD	Confirmed by spirometry indicating intermittent or chronic obstructive ventilatory pattern, FEV1/FVC<70% of expected.
Proteinuria	>0.5 gram/24hours.
Hematuria	>5 red blood cells/high power field.
Sterile pyuria	>5 white blood cells/high power field.
Cellular casts	Heme-granular or red blood cell casts.
Glomerulonephritis	verified by renal biopsy.
WHO-class	1: normal/minimal changes

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	2: mesangioproliferative GN 3: focal, segmental proliferative GN 4: diffuse proliferative GN 5: membraneous GN 6: end-stage GN
Oral or nasal ulcers	Erosions, superficial or deep, of the buccal, labial, lingual, palatal, pharyngeal, or nasal mucosa. They may be painful or painless.
Xerostomia	Oral dryness based on salivary gland destruction documented by sialometry, salivary scintigraphy or salivary gland biopsy.
Sterile peritonitis or ascites	Documented by imaging or puncture.
Intestinal vasculitis	Confirmed by abdominal angiography or histologically.
Autoimmune hepatitis	Exclusion of viral etiology and confirmed by liver biopsy.
Primary biliary cirrhosis	Elevated serum levels of alkaline phosphatase, often anti-mitochondrial antibodies. Confirmed by liver biopsy.
Celiac disease	1) evidence of malabsorption, 2) abnormal jejunal biopsy showing characteristic changes of the villi, and 3) clinical, and serological improvement after institution of a gluten-free diet.
Non-hemolytic anemia	Blood level of hemoglobin below lower normal range without signs of hemolysis. Evaluation of hemolysis may include reticulocyte count, serum levels of LDH, free hemoglobin and haptoglobin.
Immunohemolytic anemia	Blood level of hemoglobin below lower normal range and positive direct antiglobulin (Coombs') test for autoantibodies directed against the rbc membrane antigens.
Leucocytopenia	<3.000 white blood cells x 10^9 / L.
Lymphocytopenia	Below local lower normal range.
Thrombocytopenia	<100.000 platelets x 10^9 / L.
Lymphadenopathy	An enlargement of lymph nodes greater than normal for the particular region examined.
Headache	Includes: Migraine, Tension headache, Cluster headaches, Pseudotumor cerebri (benign intracranial hypertension) and Intractable non-specific headache. Lupus headache: Severe persistent headache; may be migrainous, but must be non-responsive to narcotic analgesia.
Cognitive dysfunction	The types of cognitiv deficits patients manifest include complex attention, aspects of memory (e.g. learning and recall), visual-spatial processing, language (e.g. verbal), psychomotor speed. Cognitive dysfunction can range from mild impairment to severe dementia. It represents a decline from a previously higher level of functioning and may impede social, educational or occupational functioning. Subjective complaints of cognitive dysfunction are common, although not always objectively verifiable.

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	Neuropsychological testing is the diagnostic procedure of choice for suspected cognitive dysfunction.
Aseptic meningitis	A clinical syndrome of fever, headache and meningeal irritation with CSF pleocytosis and negative cultures.
Seizures or chorea	Seizures: Abnormal paroxysmal neuronal discharge in the brain causing abnormal function. Seizures may occur with or without the loss of consciuosness. Seizures are divided in two groups, partial and generalized. Partial seizures have clinical and electroencephalographic evidence of a focal onset: the abnormal discharge usually arises in a portion of a hemisphere and may spread to the rest of the brain during a seizure. Primary generalized seizures have no interictal evidence on EEG of focal onset. A generalized seizure can be primary or secondary.
Chorea	Chorea consists of irregular, involuntary and jerky movements, that may involve any portion of the body in random sequence. Each movement is brief and unpredictable.
Peripheral neuropathy	Mononeuropathy singel/multiplex: Disturbance ot the function of one or more peripheral nerve(s). Weakness and paralysis can be due to either conduciton block in the motor nerve fibers or to axonal loss. Conduciton block is related to dymyelinisation with preservation of axon continuity. Remyelinisation can be rapid and complete. If axonal interrupeion takes place, axonal degeneration occurs below the site of interruption and recovery is ofetn slow and incomplete. Sensory symptoms and sensory loss may affect all modalities or be restricted to certain forms of sensation.
Plexopathy	A disorder of the brachial or lumbosacral plexus producing muscle weakness, sensory deficit and/or reflex change that do not correspond to the territory of a single root or nerve.
Polyneuropathy	Acute or chronic disorder of sensory and motor peripheral nerves with variable tempo characterized by symmetry of symptoms and physical findings in a distal distribution.
Autonomic neuropathy	A disorder of the autonomous nervous system which gives rise to orthostatic hypotension, sphincteric erectile/ejaculatory dysfunction, anhidrosis, heat intolerance, constipation.
Cranial nerve affection	A clinical syndrome affecting the specific sensory and/or motor function of the cranial nerve(s).
Transverse myelopathy	Disorder of the spinal cord characterized by rapidly evolving paraparesis and/or sensory loss, with a demonstrable motor and/or sensory cord level and/or sphincter involvement.
Organic brain syndrome	Altered mental function with impaired orientation, memory or other intellectual function, with rapid onset and fluctuating clinical features, inability to sustain attention to environment, plus at least two days of the following:

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	perceptual disturbance, incoherent speech, insomnia or daytime drowsiness, or increased or decreased psychomotor acitivity.
Psychosis	Severe disturbance in the perception of reality characterized by delusions and/or hallucinations.
Affective disorder	Prominent or persistent disturbance in mood characterized by either: - Depressed mood or markedly diminished interest or pleasure in almost all activities or – Elevated, expansive or irritable mood.
Thyreoditis	Diagnosis supported by thyroid scintigraphy, thyroid hormone status depends on phase of thyreoiditis.
Type 1 diabetes mellitus	Immune-mediated diabetes mellitus characterized by young age of onset, normal to wasted body habitus, low to absent plasma insulin,and high suppressible plasma glucagon.
Amenorrea	Failure of menarche by age 16 or absence of menstruation for 6 months in a woman with previous periodic menses.
>2 major infections	Note time of 3rd infection requiring hospitalization.

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Results

In **paper I**, we have focused on the analysis of the diagnostic and predictive value of anti–dsDNA antibodies, assessed by CLIFT in the mentioned patients.

In particular, we looked at the prevalence of different clinical diagnoses in CLIFT positive patients, to determine the diagnostic accuracy of the test.

The results showed limited diagnostic role played by a single assessment of anti-dsDNA antibodies, despite the high specificity of the test. The most prevalent diagnosis, in absolute and relative terms, among the 60 anti-dsDNA positive patients was SLE (24 cases). Nonetheless, in the majority of CLIFT positive patients (36 cases), many other diagnoses were found, herein non-inflammatory rheumatic diseases and non-rheumatic diseases.

Moreover, the results of simultaneous measurements were poorly reproducible, both in the inter-assay comparison between 2 different commercial kits and in the intra-assay comparison of the same kit in two different participating laboratories. The agreement of results ranged between 51% and 68%.

In addition, about one fourth of the CLIFT positive patients belonged to the ANA-negative control group, herein one SLE patient, suggesting that the negative result of preliminary ANA-screening does not rule out anti-dsDNA positivity. On the other hand, the joint positivity of ANA and CLIFT increased the specificity for SLE diagnosis up to 99%.

Table 1. Results of ANA and anti-dsDNA analyses and number of patients diagnosed with SLE at study entry.

				ANA		CLIFT A (+)		CLIFT B (+)
	No. of patients	%	SLE	(+)	(–)	Copenhagen	Tromsø	Lund
Copenhagen	138	23.6	43	69	69	20	23	23
Lund	302	51.7	11	149	153	7	7	20
Tromsø	144	24.7	11	74	70	6	6	3
Total	584	100	65	292	292	33	36	46

ANA, Anti-nuclear antibodies; SLE, systemic lupus erythematosus; CLIFT, *Crithidia luciliae* immunofluorescence test; A, ImmunoConcepts kit; B, Euroimmun kit; (+), positive; (–), negative.

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In our study, the PPV of CLIFT for SLE diagnosis ranged between 0.46 and 0.61. It is not so satisfactory, but it is important to keep in mind that SLE is a rare disease, which determines a rather low positive predictive value (PPV) even if the test performs well. Of importance is the rather high LR+, which may play a crucial diagnostic role, if the pre-test probability of having SLE is high.

Finally, the analysis of the clinical diagnoses after a 5-years follow-up showed that positivity of anti-dsDNA antibodies by CLIFT did not pose any increased risk for onset of SLE, which is in contrast with previous studies performed in more selected patients.

In summary, the diagnostic and predictive value of CLIFT-determined positivity of anti-dsDNA antibodies in patients with recent onset of rheumatic symptoms is limited, since it is found in many non-SLE patients and does not pose any increased risk for patient of being affected by SLE within 5 years. Nonetheless, anti-dsDNA antibodies assessed by CLIFT are very specific for SLE, especially in ANA positive patients. Thus, in a more general clinical contest, where symptoms and signs in patients are given the highest diagnostic relevance, anti-dsDNA antibodies may play a central role in discriminating SLE patients among unselected patients.

Table 2. Clinical diagnoses at study entry and at the last observation.

	Entry			Last observation		
		CLIFT +			CLIFT +	
Diagnosis	Total (%)	ANA +	ANA –	Total (%)	ANA +	ANA –
Systemic lupus erythematosus	65 (11.1)	23	1	65 (11.1)	23	1
Other connective tissue disease (APS; SSc; SjS; UCTD; MCTD; PM/DM)	56 (9.6)	7	1	56 (9.6)	6	1
Chronic inflammatory joint disease (RA; PsA; AS)	157 (26.9)	6	7	135 (23.1)	5	7
Inflammatory systemic disease (PMR/TA; AAV; OSV; AOSD)	29 (5)	2	1	28 (4.8)	2	1
Osteoarthritis	59 (10.1)	0	2	68 (11.6)	0	1
Arthralgia	83 (14.2)	0	2	36 (6.2)	0	0
Soft tissue rheumatism	36 (6.2)	1	0	34 (5.8)	1	0
Dermatological disorder (DLE; CV)	18 (3.1)	1	0	17 (2.9)	1	0
Non-rheumatic disorder	63 (10.8)	4	1	131 (22.4)	6	4
No diagnosis	18 (3.1)	0	1	5 (0.9)	0	0
Drop-out	–	–	–	6 (1)	0	1
Healthy	–	–	–	3 (0.5)	0	0

CLIFT, *Crithidia luciliae* immunofluorescence test; ANA, anti-nuclear antibodies; APS, anti-phospholipid syndrome; SSc, systemic sclerosis; SjS, Sjögren's syndrome; UCTD, undifferentiated connective tissue disease; MCTD, mixed connective tissue disease; PM/DM, polymyositis/dermatomyositis; RA, rheumatoid arthritis; PsA, psoriatic arthritis; AS, ankylosing spondylitis; PMR/TA, polymyalgia rheumatica/temporal arteritis; AAV, anti-neutrophil cytoplasmic antibody (ANCA)-associated vasculitis; OSV, other systemic disease; AOSD, adult-onset Still's disease; DLE, discoid lupus erythematosus; CV, cutaneous vasculitis.

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The article describing this part of the project, entitled "Low diagnostic and predictive value of anti-dsDNA antibodies in unselected patients with recent onset of rheumatic symptoms: results from a long-term follow-up Scandinavian multicentre study", was published in 2013 and is included as "paper I" in the appendix of this thesis.

Table 3. Specificity (Sp), sensitivity (Se), positive predictive value (PPV), and likelihood ratio of positive (LR+) and negative (LR–) results for clinical diagnosis of SLE.

Assay	Sp	Se	PPV	LR+	LR–
ANA	0.55	0.91	0.2	2	0.2
CLIFT A Copenhagen	0.97	0.31	0.61	10.3	0.7
CLIFT A Tromsø	0.96	0.28	0.5	7	0.75
CLIFT B	0.97	0.32	0.46	10.7	0.7

ANA, Anti-nuclear antibodies; CLIFT, *Crithidia luciliae* immunofluorescence test.

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In **paper II**, we have analyzed whether the positivity of anti-dsDNA antibodies is associated with any particular clinical phenotype, regardless of diagnosis.

We performed simultaneously a total of seven (3 CLIFT and 4 ELISA) assessments of anti-DNA antibodies, in aliquots of serum from the recruited patients in three different labs, and characterized patients´ clinical phenotypes as described above.

The investigation has revealed that the assessment of anti-dsDNA antibodies with different techniques results in a considerable discrepancy of outcomes and of correlations to various clinical and biochemical manifestations.

The positivity of anti-dsDNA antibodies by IIF (CLIFT) and Enzyme Linked Immunosorbent Assay (ELISA) displays a significant association with proteinuria and pleuritis. Alopecia has significant association with CLIFT determined anti-dsDNA antibodies.

Table 5 Association between most relevant clinical manifestations and positive outcome of anti-dsDNA tests (any CLIFT and any ELISA)

	Any CLIFT positive (39 ANA positive+20 ANA negative)		Any ELISA positive (50 ANA positive+49 ANA negative)	
	CRUDE OR (95% CI)	ADJUSTED OR (95% CI)	CRUDE OR (95% CI)	ADJUSTED OR (95% CI)
Peripheral arthritis	1.1 (0.6–2)		0.6 (0.4–1.04)	
Photosensitivity	0.6 (0.2–1.7)		0.5 (0.2–1.2)	
Oral ulcers	0.8 (0.25–2.9)		1.8 (0.8–4.1)	
Haematuria	3.8 (1.4–10.4)	0.6 (0.1–3.4)	3.2 (1.3–8)	0.5 (0.1–2.8)
Proteinuria	14 (5.1–38.4)	13 (2.9–57.7)	16.7 (5.3–52.6)	18.8 (3.7–95.2)
Malar rash	3.1 (0.97–10.2)		1.7 (0.5–5.4)	
Anaemia	3.8 (1.4–10.4)		1.1 (0.4–3.5)	
Leukopenia	6.8 (2.3–20.3)	2.3 (0.5–10.7)	6.5 (2.2–19.1)	3.5 (0.8–14.2)
Alopecia	4.4 (1.3–15)	4.3 (1.1–16)	3.35 (1–10.8)	3.1 (0.9–10.8)
Lymphopenia	10.9 (3.2–37)		2.7 (0.8–9.2)	
Discoid LE	1.2 (0.1–9.8)		0.6 (0.8–5.3)	
Thrombocytopenia	8.8 (2.1–36.2)	3.1 (0.5–20.2)	4.7 (1.1–19)	1.1 (0.2–7.6)
Pleuritis	13 (2.1–79.6)	11.1 (1.5–83.8)	18.8 (2.1–170.3)	14.5 (1.4–148.2)
Arthralgia	0.6 (0.3–1.02)		0.5 (0.3–0.8)	
Morning joint stiffness	0.5 (0.2–1.04)		0.4 (0.2–0.7)	
Raynaud's phenomenon	1.6 (0.8–3.2)		1.2 (0.6–2.2)	
Headache	0.7 (0.3–1.75)		0.8 (0.4–1.6)	
Xerostomia	0.75 (0.3–1.8)		1 (0.5–2)	
Arterial hypertension	0.96 (0.4–2.2)		1 (0.5–1.9)	
Tendinitis	0.2 (0.02–1.2)		1.3 (0.7–2.7)	
Psoriasis	0.6 (0.2–2.2)		0.5 (0.2–1.4)	
Affective disorder	0.7 (0.2–2.2)		0.8 (0.3–1.9)	
Keratoconjunctivitis sicca	0.4 (0.1–1.8)		0.8 (0.3–2)	
Cutaneous vasculitis	6.7 (2.25–20.2)	2.1 (0.5–9.4)	6.4 (2.2–18.9)	2.4 (0.6–9.5)
Asthma or COL	2.4 (1.04–5.5)		1.4 (0.7–3.15)	
Lymphadenopaty	6.5 (1.4–29.7)		1.8 (0.3–9.6)	

Crude OR with corresponding 95% CIs in brackets is reported for all the variables. Adjusted OR is reported only for variables significantly associated with positivity of any CLIFT and any ELISA.
ANA, antinuclear antibodies; CLIFT, Crithidia Lucillia Immunofluorescence Test.

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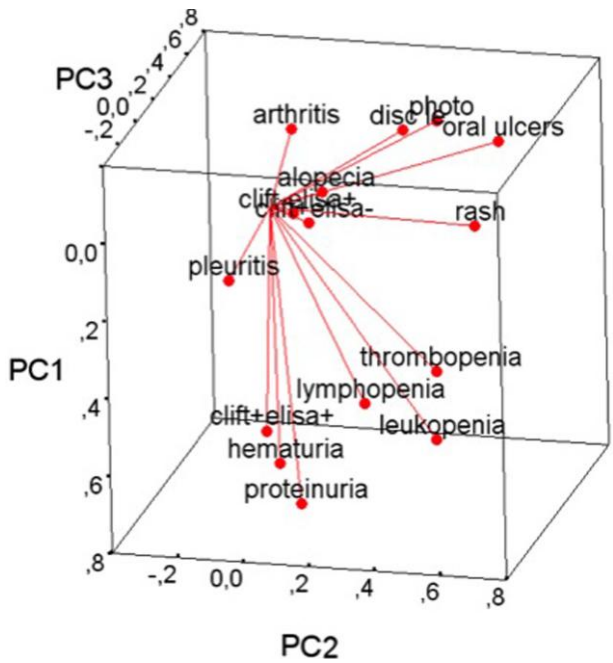
In distinct subgroups of patients, anti-dsDNA antibodies are also variously associated with presence of other clinical manifestations, such as cutaneous vasculitis, hematuria, leukopenia and thrombocytopenia, which were not confirmed after multivariate analysis. Principal components analysis indicated that joint positivity of CLIFT and ELISA clusters with nephropathy, hematologic abnormalities and pleuritis.

In summary, positivity of anti-dsDNA antibodies, independently of the laboratory method used for their assessment is associated with presence of proteinuria and pleuritis, regardless of the clinical diagnosis and the outcome of ANA screening.

Our results may shift the importance of anti-dsDNA antibodies from the well-known role as diagnostic hallmark for SLE to a crucial pathogenic factor in the manifestation of the above mentioned clinical features, independently of the diagnosis.

The article ”Clinical phenotype associations with various types of anti-dsDNA antibodies in patients with recent onset of rheumatic symptoms. Results from a multicentre observational study” was published in 2014 and is included as “paper II” in the appendix.



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Part two: Novel potential biomarkers (papers III and IV)

A common approach to the search for novel biomarkers is to look at known or suspected pathogenic mechanisms. Among the different cells involved in the pathogenesis of SLE, the crucial role played by PMNs and pDCs has recently been emphasized. Moreover, the immune complexes mostly contain nuclear material, able to stimulate immune cells through TLR7 and TLR9, and are formed upon binding of autoantibodies to different antigens, often remnants of dying cells due to impaired clearance of apoptotic cells [295]. The role of cytokines is also important for the up- and down-regulation of many processes involved in the pathogenesis.

Based on previous studies from our group and data published by other researchers, we have chosen to draw our attention to three potential biomarkers, namely serum-induced phagocytosis of necrotic cell material by PMNs (paper III), Osteopontin (paper IV) and S100A8/A9 (paper IV). In particular, we have focused on the study of the temporal associations between outcome of these potential biomarkers and SLE-related clinical features and disease activity.

Study design

To look for novel biomarkers for a certain group of patients implies the need to study a well-selected population of patients affected by the disease, preferably prospectively and longitudinally.

In the second part of the present research project, we focused on a population of patients affected by established SLE, who participated in a prospective follow-up program at the Rheumatology clinic, University Hospital in Lund, referred to as NuLAS (Nurse Lupus Activity Screening), approved by the local ethical committee (project no. LU 378-02).

The general purpose of NuLAS was to improve care of SLE patients and to identify clinical and laboratory variables that could be considered as markers or predictors of complications and exacerbations of the disease. The patients were followed longitudinally for more than two years with periodically scheduled visits every 60±20 days. An extensive set of clinical and laboratory variables were registered in a database tailored for the study. Serum samples were collected also before and after the date of the clinical assessment when needed.

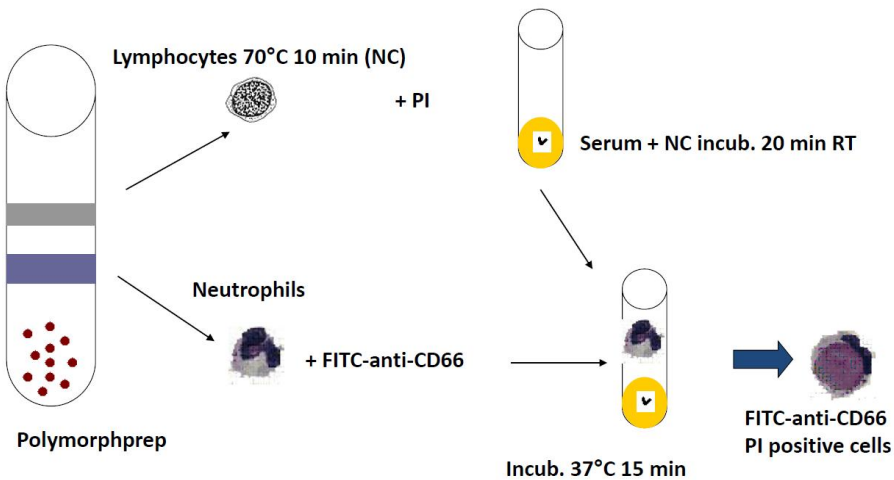
Assessment of biomarkers

PNC assay or assay for serum-induced phagocytosis of necrotic cell material (paper III). It is a flow cytometry-based assay, with some modifications to the one originally developed in 2004 as an *in vitro* assessment and quantification of LE cells in patients with suspected SLE[344].

As schematized in the figure below, PMNs and peripheral blood mononuclear cells (PBMCs) are isolated from heparinized blood using Polymorphprep™ (Axis-Shield Poc AS, Oslo, Norway), as recommended by the manufacturer. To obtain necrotic cell material (NCM), PBMCs are incubated for 10 min at 70°C and the NCM is stained with propidium iodide (PI) (BD Biosciences Pharmingen, San Diego, CA, USA). PMNs are stained with anti-CD66-FITC antibodies (Dako A/S, Glostrup, Denmark).

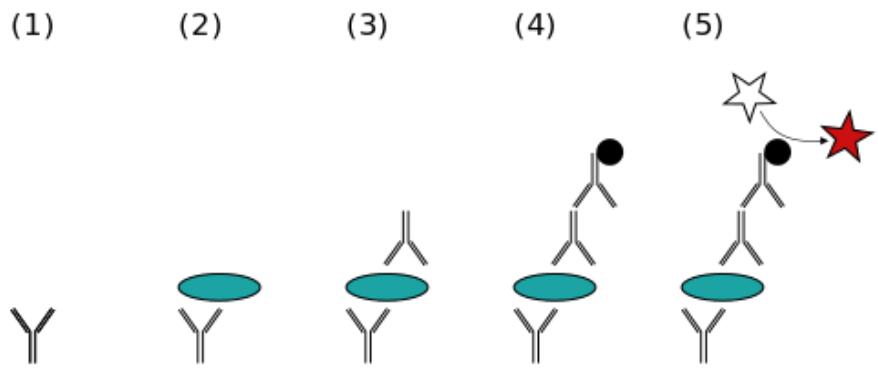
For autoantibody binding and complement activation, PI-labeled NCM (4.5 x 105 cells) is incubated with 30 µl undiluted serum at room temperature for 20 min, followed by addition of PMNs isolated from healthy individuals (0.3 x 106 cells in a total volume of 300 µl) for another incubation at 37°C for 15 min. Cells are washed with phosphate buffered saline (PBS) pH 7.2 containing 0.1% human serum albumin (Sigma-Aldrich St. Louis, MO, USA) before analysis by flow cytometry. PMNs are identified based on forward and side scatter properties and by computerized gating. Phagocytosis is calculated from the percentage of cells positive for both CD66 and PI (% $CD66^{+}PI^{+}$ PMNs).

Reference values were obtained from 148 apparently healthy individuals.



Measurement of P-OPN (paper IV). Plasma levels of OPN were measured by an ELISA commercial kit (Quantikine® - R&D Systems, Inc, Minneapolis, USA) according to the manufacturer's instructions. The assay employs the quantitative sandwich ELISA technique with microplate coated with a monoclonal antibody against human OPN. Plasma levels over 150 ng/ml (corresponding to mean value + 2SD in apparently healthy controls, as reported by the manufacturer) were referred to as high.

Measurement of S-S100A8/A9 (paper IV). Serum samples were added to microtitre plates (Maxisorp, Nunc, Roskilde, Denmark) coated with monoclonal antibody MRP8/14 (27E10, BMA Biomedicals, Augst, Switzerland) and incubated before biotinylated polyclonal antibodies against S100A8/A9 (chicken polyclonal antibody MRP8/14, Abcam, Cambridge, UK) were added. This step was followed by adding ALP-labeled streptavidin (Dako, Glostrup, Denmark). After incubation, bound streptavidin was visualized by adding disodium-*p*-nitrophenyl phosphate (Sigma, St Louis, MO, USA) 1 mg/ml dissolved in 10% (w/v) diethanolamine pH 9.8 containing 50mM $MgCl_2$ and the absorbance was measured at 405 nm. The values reported are means of duplicates with subtracted background, obtained using uncoated wells. The concentrations were calculated from titration curves obtained from one serum with known concentration. The lower detection level of S100A8/A9 was 3 ng/ml and serum levels over 44 µg/ml (corresponding to mean value + 2SD in 79 matched apparently healthy controls) were referred to as high.



A sandwich ELISA. (1) Plate is coated with a capture antibody; (2) sample is added, and any antigen present binds to capture antibody; (3) detecting antibody is added, and binds to antigen; (4) enzyme-linked secondary antibody is added, and binds to detecting antibody; (5) substrate is added, and is converted by enzyme to detectable form.

By Jeffrey M. Vinocur (Own work), via Wikimedia Commons

Other assessments

Routine laboratory tests were used for the longitudinal measurement of all the variables needed to assess the disease activity according to SLE disease activity index 2000 (SLEDAI-2K) [65], including the complement components C1q, C3 and C4.

A semi-quantitative assessment of anti-dsDNA antibodies, using CLIFT, and ELISA measurement of antibodies against a mixture of histone proteins were performed.

Disease activity was assessed every time, using the SLEDAI-2K[65]. For the assessment of relationships with outcome of PNC assay in combination with other biomarkers, a modified version of SLEDAI-2K was used, excluding any score given for low levels of complement factors and/or anti-dsDNA antibodies.

Patients and control groups

A total of 69 patients (referred to as SLE group Lund) affected by SLE, fulfilling ACR 1982 classification criteria for SLE[61], and participating in the previously described NuLAS, were included in the investigation for the longitudinal assessment of PNC assay (paper III).

Since we aimed at verifying its diagnostic accuracy, we performed a cross-sectional PNC assay also in 148 apparently healthy volunteers and in 529 patients (referred to as multicentre rheumatic group or MRG) with recent onset of any rheumatic disease, recruited in three different centres, Copenhagen in Denmark (138 patients), Lund in Sweden (269 patients) and Tromsø in Norway (122 patients).

MRG patients were grouped according to initial clinical diagnoses, formulated as already described in the previous section, about the Scandinavian anti-DNA study.

We focused here on the outcome of the test in the subgroup of patients later diagnosed with SLE, in comparison with other groups of rheumatic diseases.

In paper IV, we report the results of performed repeated measurements of P-OPN and S-S100A8/A9. We investigated 59 SLE patients, participating in NuLAS, focusing on the temporal associations between SLE-related clinical manifestations and increased levels of the two potential biomarkers.

Statistical analysis

Descriptive statistics was calculated. Chi-square test and Kruskal-Wallis test were used for analysis of non-parametric data. Correlation was assessed using Spearman´s test. Agreement of results was evaluated using kappa statistics.

Temporal associations between outcome of the biomarkers and relevant clinical features were evaluated with generalized linear mixed effects model (Proc GENMOD). Odds ratios (OR), 95% confidence intervals (CI) and statistical significance levels (p-value) were calculated. A p-value ≤ 0.05 defined statistical significance. Only visits within intervals of 60±20 days were considered for temporal associations.

In paper IV, cutoff values for the assays used to discriminate between patients according to outcome were determined with Youden's index, after calculating the area under the receiver operating characteristic (ROC) curve (AUC).

The Software SAS 9.3 and IBM SPSS Statistics 20.0 were used for all statistical analyses.

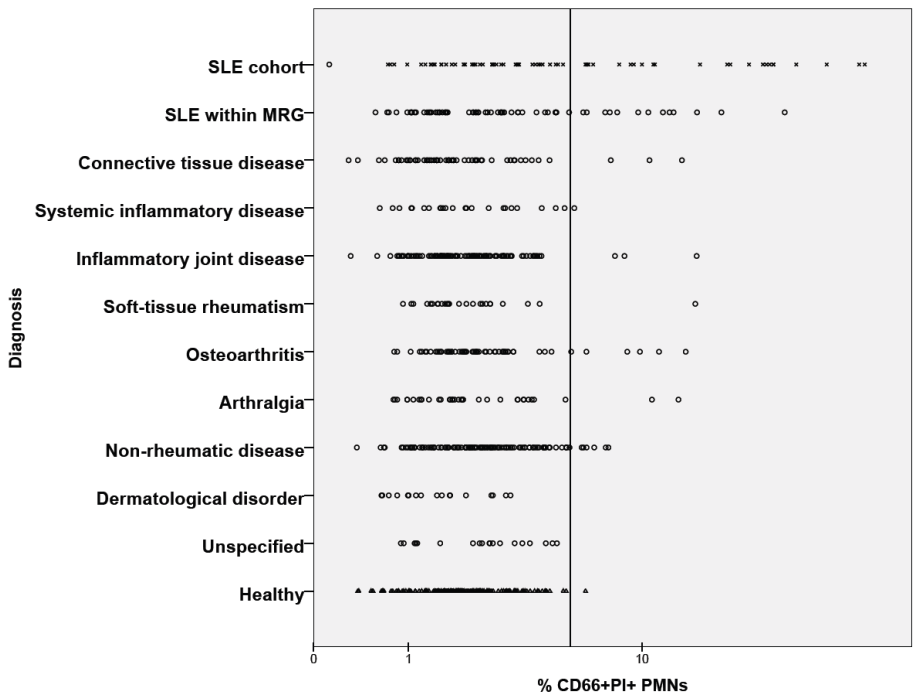
Demographics and results of PNC assay in healthy controls, multicenter rheumatic group and SLE group.

	Healthy (n=148)	Multicentre rheumatic group (n=529)		SLE group (n=69)
		Other diagnoses (n=464)	SLE (n=65)	
Age (years)				
Mean (SD)	41.1 (13.1)	51.8 (15.4)	39.7 (16.1)	41.4 (13.7)
Median (range)	40 (19-74)	53.2 (15-84)	35 (15-75)	39.2 (18-76)
Gender				
Females (%)	127 (85.8)	367 (78.9)	61 (93.8)	63 (91.3)
$CD66^+PI^+$ PMNs (%)			*	* #
Mean (SD)	2.1 (1.1)	2.5 (2.0)	4.3 (5.0)	7.3 (9.0)
Median (range)	1.9 (0.4-6.3)	2 (0.3-15.4)	2.6 (0.6-30.2)	3.4 (0.8-49.9)

$CD66^+$ PI^+ PMNs: double positive polymorphonuclear cells, flow cytometry analysis
*= significant difference ($p<0.05$) compared to healthy
#= significant difference ($p<0.05$) compared to rheumatic group with other diagnoses

Results

In **paper III**, our cross-sectional analysis demonstrated that positivity in PNC assay is specific but not exclusively found in SLE patients. It was detected in 6.6% of patients with recent onset of any rheumatic symptoms, whereof the majority were non-SLE patients. Based on these results we could estimate for PNC assay 0.20 sensitivity, 0.95 specificity and 0.37 positive predictive value (PPV) for SLE diagnosis. The simultaneous positivity of CLIFT and PNC assay was mostly detected in SLE patients, increasing the specificity up to 0.99 and the PPV for SLE diagnosis up to 0.66.



Outcome of PNC assay in the different groups of participants in the study.
The vertcal line represents the threshold between normal and pathologic values.

In the longitudinal assessment of 1100 sera collected over time in SLE patients, positive outcome of the PNC assay was documented 368 times, totally, and at least once in more than 60% of patients. In many patients, the outcome of the PNC assay varied during the follow-up period. In general, we could observe that events of nephritis, hypocomplementemia, and presence of antibodies to dsDNA and histone proteins were more prevalent in the SLE patients with redundant positive PNC assay. In the SLE patients with recurrent negative outcome of PNC assay, arthritis was more prevalent and hematologic manifestations were rarer than the rest of the SLE cohort.

Prevalence of clinical manifestations in subsets of patients within SLE group, according to the outcome of PNC assay over time.

Clinical manifestation	Always positive PNC	Always negative PNC	Variable PNC
	12 patients (166 assessments)	31 patients (475 assessments)	26 patients (459 assessments)
Glomerulonephritis	31.9%	10.5%*	6.5%*
Arthritis	1.8%^	16.6%	6.1%*^
Mucocutaneous	24.7%	21.3%	13.9%*^
Alopecia	10.8%	7.8%	7.2%
Hematologic	3.0%#	1.1%#	7.0%^
SLEDAI – 2K >0	57.8%	48.8%	44.7%*^

* = significant difference ($p \leq 0.05$) compared to “Always positive PNC”
^ = significant difference ($p \leq 0.05$) compared to “Always negative PNC”
# = significant difference ($p \leq 0.05$) compared to “Variable PNC”

Significant correlations with active hematologic involvement, such as leukopenia and thrombocytopenia, and upcoming mucocutaneous manifestations were found. No significant temporal relationships between outcome of PNC assay and increased SLEDAI score were found.

The simultaneous presence of anti-dsDNA antibodies and positive PNC assay showed no association with any current or upcoming clinical manifestation.

Positivity of anti-histone antibodies, in combination with positive PNC test could predict future mucocutaneous manifestations. The combination of positive PNC assay with low levels of C1q showed a strong relationship with lupus nephritis. When PNC positivity is combined with low levels of C3 significant relationships with nephritis, alopecia and increased modified SLEDAI-2K are documented.

In summary, PNC assay is a laboratory tool that can be used in patients with established SLE, preferably in combination with anti-histone antibodies and, above all, complement factors, with potential to predict important clinical features. Nonetheless, the diagnostic value of PNC assay and its power to discriminate SLE patients among patients with recent onset of rheumatic symptoms is limited.

The article ”Testing phagocytosis of necrotic material by polymorphonuclear cells predict clinical manifestations in Systemic Lupus Erythematosus: an observational study” was submitted for publication in 2015 and is included as “paper III” in the appendix.

In **paper IV**, the longitudinal analysis of OPN and S100A8/A9 in 973 blood samples demonstrated that these markers might play a potential role as supplementary laboratory tools in the management of SLE patients.

In fact, increased levels of P-OPN and S-S100A8/A9 were found at least once in 11 and 38 patients, respectively. Weak correlation and low agreement of results between P-OPN and S-S100A8/A9 were found. In many patients, the outcome of the measurements varied during the follow-up period.

High P-OPN was generally associated with current and upcoming glomerulonephritis. AUC for this manifestation was 0.67 (0.61-0.74). A cutoff level of 74 ng/ml predicted glomerulonephritis with specificity (Sp) 0.80 and sensitivity (Se) 0.50.

Raised serum levels of S100A8/A9 were associated with current active glomerulonephritis and arthritis, and were able to predict the occurrence of the arthritis and mucocutaneous manifestations within 2 months. AUC for current glomerulonephritis was 0.66 (0.61-0.71). A cutoff level of 12.4 µg/ml predicted glomerulonephritis with Sp 0.75 and Se 0.41.

A significant relationship with present and future increased SLEDAI-2K was found for increased levels of both P-OPN and S-S100A8/A9.

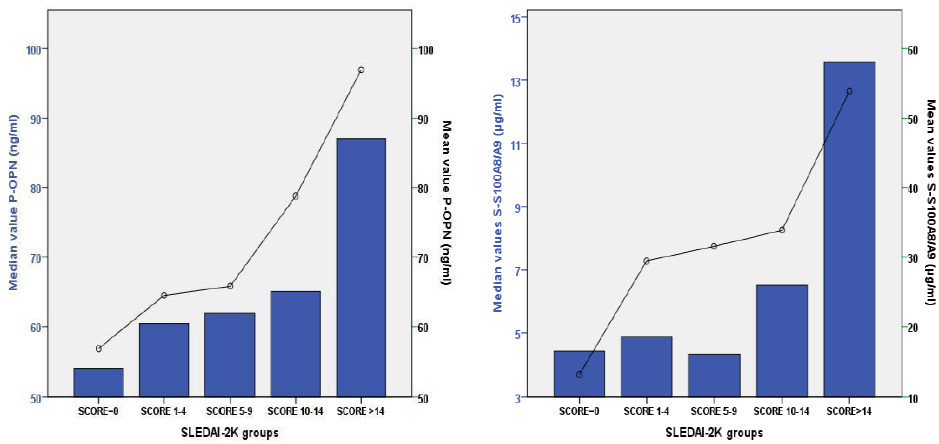
In contrast to previous reports, in our SLE group we did not find many patients with high levels of P-OPN. It could be explained by the fact that we calculated the highest reference value according to the mean value (94.8 ng/mL) and standard deviation (24.9 ng/mL) provided by the manufacturer of the commercial ELISA-kit used in our study, in order to test the usefulness of the test in a realistic clinical setting. A pilot test of the same assay performed in our control group of healthy individuals displayed much lower P-OPN values (data not shown). It should lower the highest reference value to around 80 ng/mL, which is much closer to the cutoff value (74 ng/mL) for prediction of glomerulonephritis. These data await verification and, if confirmed, they would require a re-assessment and revision of the described relationships between P-OPN and clinical features in SLE patients.

The article “Osteopontin and S100A8/A9 as potential biomarkers in Systemic Lupus Erythematosus: an observational longitudinal study” is presented as manuscript and is included as “paper IV” in the appendix.

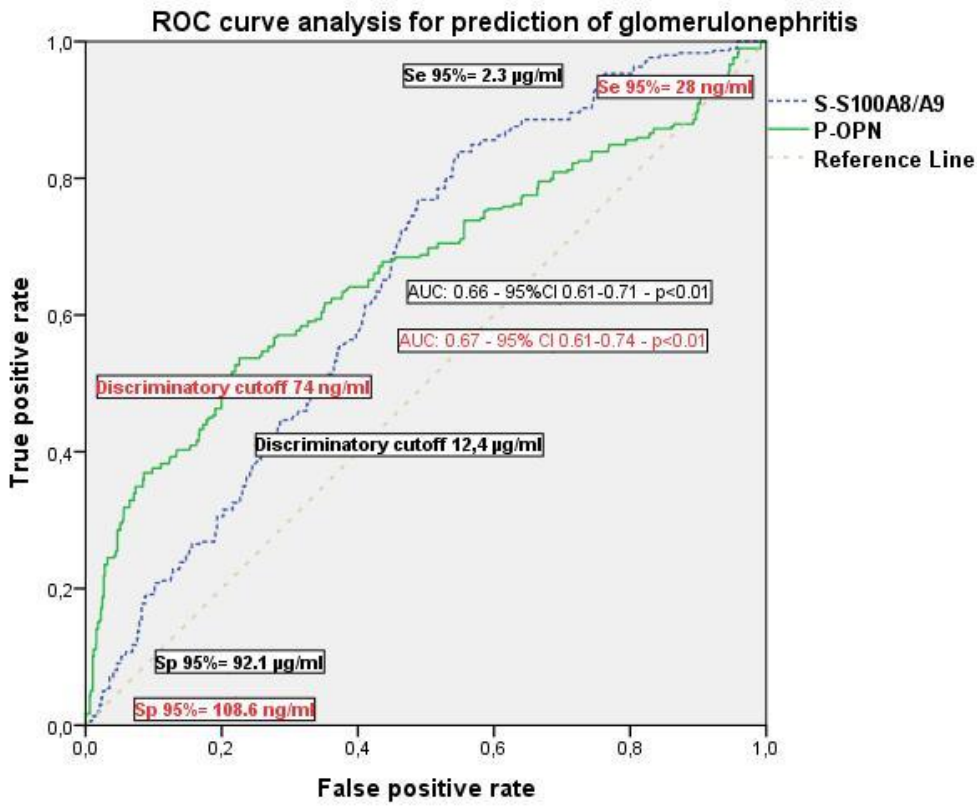
Prevalence of clinical manifestations grouped according to the outcome of P-OPN and S-S100A8/A9 over time (973 events).

Outcome of test	No of events	Glomerulonephritis	Arthritis	Mucocutaneous	Alopecia	Hematologic	SLEDAI-2K >0
High P-OPN	19	47.4%*	5.3%	26.3%	21.1%	5.3%	73.7%*
Normal P-OPN	954	10.2%	10.0%	18.1%	8.1%	4.0%	47.8%
High S-S100A8/A9	122	19.7%*	9.8%	17.2%	9.8%	1.6%	55.4%
Normal S-S100A8/A9	851	9.6%	9.9%	18.4%	8.1%	4.3%	47.3%
Total	973	10.8%	9.9%	18.3%	8.3%	4.0%	48.3%

*Significant difference ($p<0.05$) compared to group with normal levels



Median (bars) and mean (line) values of P-OPN and S-S100A8/A9 in SLE patients grouped according to the SLEDAI-2K score.



ROC curve analysis for prediction of glomerulonephritis.
Cutoff values and AUC for P-OPN and S-S100A8/A9 are reported in red and black text, respectively**.**

Conclusions and future perspectives

The results of the present research project may be summarized in the few following sentences.

Neither anti-dsDNA antibodies nor the other analyzed potential biomarkers fulfill the criteria for being considered ideal biomarker or surrogate endpoint for SLE as a whole.

Testing for anti-dsDNA antibodies in the diagnostic process is not as crucial as generally believed, at least in a realistic diagnostic scenario.

The outcome of tests used for the assessment of anti-dsDNA antibodies is poorly reproducible, showing wide variability in both inter-assay and intra-assay analyses.

Anti-dsDNA antibodies may rather represent a marker that mirrors current presence of certain clinical features, such as nephritis and pleuritis, regardless of the diagnosis.

PNC assay, P-OPN and S-S100A8/A9 may play a role as supplementary laboratory tools in the management of SLE patients, but their validation as reliable biomarkers needs to be further investigated in large scale multicentre SLE cohorts.

The different relationships between the analyzed biomarkers and the fluctuation of SLE disease activity may be an indication confirming their pathogenic role.

In other words, PMNs, along with anti-histone antibodies and functional complement system, as well as the pleiotropic proteins OPN and S100A8/A9 interplay somehow and are involved, upstream or downstream, in different steps of the long and complex stairway to the SLE syndrome.

It may represent the ramp for launching novel approaches in therapeutic research, focusing on their biologic blockade or modulation.

Populärvetenskaplig sammanfattning.

SLE är förkortningen för Systemisk Lupus Erythematosus, en reumatisk systemsjukdom med okänd orsak. Både ärftliga och miljömässiga faktorer är viktiga för utvecklingen av sjukdomen. SLE har definierats som ett syndrom, eftersom det kan tolkas som en blandning av flera olika sjukdomar, som påverkar många kroppsdelar, såsom rörelseapparaten, hud och inre organ. Olika patienter som delar diagnosen SLE har ofta olika symptom och besvär, med olika behandlingar och prognos som följd. Mildare varianter av SLE kännetecknas ofta av ihållande inflammation i hud och slemhinnor och/eller i leder, medan mer aggressiva typer av SLE innebär medverkan av njurar och nervsystem, vilket ofta leder till tyngre behandling och ökad risk för livshotande sjukdomsrelaterade skador.

Det saknas nuförtiden tillförlitliga SLE biomarkörer, det vill säga labprov som kan vara vägledande då man ska ställa diagnos, under en pågående plötslig försämring p.g.a. att SLE blivit mer aktivt (så kallat ”skov”) eller då man behöver utvärdera resultatet av någon pågående behandling.

Trots många förmodade biomarkörer har ingen av dem validerats hittills. Den största utmaningen i att identifiera och utveckla specifika biomarkörer för SLE är den komplexa bakomliggande orsaken och det varierande sjukdomsuttrycket.

SLE-patienter har ofta avvikande immunsystem, vilket resulterar i, bland annat, produktion av felaktiga antikroppar som skadar olika kroppsvävnader.

Närvaron i blodet av anti-dubbelsträngade (ds) DNA-antikroppar har ofta varit starkt förknippad med SLE. Anti-dsDNA antikroppar anses ganska specifika för SLE och ofta som en riskfaktor för förekomst av njurskada, därmed sämre prognos och kortare överlevnad.

Man har tidigare ifrågasatt antikropparnas värde som tillförlitlig markör av SLE, eftersom det finns personer som aldrig blir sjuka trots att man finner anti-dsDNA antikroppar i deras blod. Många SLE patienter resulterar negativa vid upprepade kontroller av antikropparna, men kan drabbas hårt av sjukdomen i alla fall.

I denna avhandling syftar jag till att undersöka användbarheten av traditionella och etablerade, såväl som nya och lovande biomarkörer vid SLE.

Resultatet av mina undersökningar visar att anti-dsDNA antikroppar inte är så avgörande som allmänt förmodat, åtminstone för att upptäcka SLE hos patienter i ett tidigt skede av sjukdomen. Anti-dsDNA antikroppar finns hos patienter med flera andra reumatiska och icke reumatiska sjukdomar. Enbart enstaka av dessa patienter utvecklar SLE inom loppet av 5 år.

Att mäta anti-dsDNA antikroppar samtidigt på olika laboratorier dit man har skickat blod tappat från samma patient kan leda till olika och motsägande resultat. Skillnaden är mer markant när man använder olika mätningsmetoder, men det finns kvar i mindre utsträckning då man på olika ställen använder samma test med snarlik utrustning.

Att finna en ökad mängd av anti-dsDNA antikroppar i blodet förefaller ha samband med närvaron av specifika kliniska problem, såsom njurpåverkan, brist på olika blodkroppar, eller lungsäcksinflammation, snarare än att vara associerad med närvaron av själva SLE sjukdomen. Dessa fynd skulle kunna betona sambandet mellan antikropparna och särskilda symptom, oberoende av diagnosen.

Anti-dsDNA antikroppar är inblandade i det så kallade Lupus Erythematosus (LE) cellfenomen, det vill säga det allra första specifika laboratoriefyndet vid SLE, upptäckt 1948. Det visade sig, vid senare undersökningar, att fenomenet beror på att en särskild typ av vita blodkroppar har ätit upp rester av döda celler.

SLE patienterna har tydligen nedsatt förmåga att bortskaffa sina celler som varje dag dör och ersätts av nya celler, vilket skapar en del obalans i immunsystemet, som exempelvis ökat antal vita blodkroppar som äter upp cellresterna.

Att leta efter LE-cellfenomenet i mikroskopet används inte längre som rutinmässig laboratorieundersökning, framförallt på grund av att det är tidskrävande.

En del av min forskning handlar om PNC-testet. Det är en relativt modern undersökning som snabbt kan mäta antalet markerade vita blodkroppar som har ätit upp markerade cellrester, liksom LE-cellfenomenet. Resultaten visar att blodbrist och tillkomst av hudutslag inom loppet av 2 månader är vanligare hos SLE patienterna med positivt PNC-test. Njurinflammation och mer aktivt SLE förekommer betydligt oftare om positivt PNC-test samtidigt kombineras med positivitet av andra traditionella blodprov, främst komplementfaktorer.

En del av nämnda vita blodkroppar kallas för neutrofiler och betraktas nuförtiden som en celltyp som är involverad i många mekanismer som ligger bakom SLE. Neutrofiler innehar flera kärnor med omgivande material som till cirka hälften består av S100A8/A9, ett protein som även känt som kalprotektin. Det är ett ämne som vanligtvis mäts i avföringen och används som biomarkör vid kronisk tarminflammation.

Neutrofiler hos en del SLE patienter har avvikande storlek och funktion, vilket kan leda till förhöjd utsöndring av S100A8/A9. Redan i början av 1990-talet, har bevis om förhöjd blodnivå av kalprotektin hos patienter med SLE publicerats. Exakt vilken roll kalprotektin spelar vid SLE har inte studerats ingående än, och dess mätning har aldrig införts i vanlig utredning eller uppföljning av sjukdomen.

I det sista delarbetet i min avhandling redovisar jag vilket samband finns mellan blodnivåer av S100A8/9 och olika kliniska fynd hos SLE patienter. Förhöjt kalprotektinvärde i blodet har tidssamband med njur- och ledinflammation och kan förutspå tillkomst av ökad sjukdomsaktivitet.

Snarlika fynd konstateras vid mätning i blod av ett annat protein som, för snart 30 år sedan, döptes till Osteopontin av den kända Lunda-professorn Dick Heinegård. Osteopontin beskrevs då som ett överbryggande ämne (”pons” betyder bro på latin) mellan olika benstrukturer (därmed ”osteo”). Många flera andra funktioner av Osteopontin har upptäckts sedan dess. Osteopontin är involverat i ett antal immunologiska mekanismer som man tror kan leda till SLE. Våra mätningar med jämna mellanrum hos patienter med SLE visar att sjukdomen blir mer aktiv och njurinflammationen tilltar i samband med eller 2 månader efter stigande Osteopontins värde i plasma.

Sammanfattningsvis, innebär dessa resultat att genom PNC-test eller mätning av S100A8/A9 och/eller Osteopontin skulle man kunna förutspå förekomst av njurinflammation eller hudutslag eller ledinflammation eller att SLE kommer att bli mer aktivt inom några veckor.

Dessa markörer behöver dock testas hos ett större antal patienter för att kunna få någon bekräftelse kring den äkta betydelsen av PNC-test, S-100A8/A9 och Osteopontin vid hantering av SLE i alla sina olika aspekter.

Varken anti-dsDNA antikroppar eller någon annan enskild laboratorieanalys får betraktas vara som guldstandard för att ställa diagnosen SLE.

Jakten på den perfekta biomarkören är inte över ännu. I avsaknad av enskilda tillförlitliga markörer som med säkerhet kan informera läkaren om patientens diagnos och sjukdomens aktivitetsnivå, får man fortsatt lita på en kombination av olika markörer och, i främsta rummet, på läkarens erfarenhet, skicklighet och förmåga att på rätt sätt sätta ihop alla bitar som komponerar SLE-puzzlet.

Riassunto

Il lupus eritematoso sistemico (LES) è una malattia reumatica sistemica autoimmune con eziologia sconosciuta, dove i fattori sia genetici che ambientali sono importanti per lo sviluppo della malattia. Anche fattori perinatali, come esposizione della madre a farmaci e sostanze chimiche, o alterato equilibrio ormonale o del sistema immunitario possono svolgere un ruolo importante per lo sviluppo futuro del LES.

Il LES è stato definito come una sindrome che colpisce molti dei sistemi dell´organismo, come il sistema muscolo-scheletrico, la pelle e gli organi interni. Si è spesso visto che pazienti che hanno in comune la diagnosi LES hanno in realtà manifestazioni cliniche molto differenti, alcune delle quali possono anche appartenere ad altre malattie. Ciò determina un diverso trattamento terapeutico e una prognosi differente. Le forme cliniche di LES più lievi sono caratterizzate spesso da infiammazione mucocutanea e/o articolare cronica. Al contrario, le varianti più aggressive implicano il coinvolgimento di organi vitali, quali i reni e il sistema nervoso centrale, il che spesso determina un trattamento farmacologico più pesante e un aumentato rischio di malattie associate, nonché di complicanze letali.

Per quanto riguarda le caratteristiche immunologiche, i pazienti affetti da LES hanno spesso una risposta immunitaria aberrante contro i cosiddetti autoantigeni, con conseguente produzione di anticorpi diretti contro cellule e tessuti propri.

I linfociti B sono iperattivi e responsabili della produzione di tali autoanticorpi, che legandosi saldamente ai rispettivi autoantigeni, determinano la formazione di immunocomplessi, che si depositano sui tessuti, danneggiandoli e compromettendone la funzionalità.

Tale processo, noto sotto il nome di autoimmunità, è importante nella patogenesi del LES, soprattutto in presenza di autoanticorpi diretti contro particolari componenti del nucleo cellulare, quali nucleosomi, il DNA nativo a doppia elica (dsDNA) e gli istoni.

La creazione di molti autoantigeni nel LES deriva dal malfunzionamento dei sistemi che regolano la morte cellulare, in particolare l´apoptosi (o morte cellulare programmata) e la netosi (cioè la morte dei globuli bianchi neutrofili attivati). Se la morte cellulare programmata non funziona regolarmente, le cellule moribonde che non vengono eliminate vanno in necrosi, con fuoriuscita di materiale intra-

cellulare e nucleare che viene riconosciuto come estraneo e quindi attaccato dal sistema immunitario tramite produzione di anticorpi e attivazione dei processi flogistici. Analogo discorso vale per i detriti cellulari generati dal malfunzionamento della netosi. Ciò che peggiora ulteriormente il quadro immunologico nei pazienti affetti da LES è l´incremento dei processi di apoptosi e netosi, che si aggiunge ad una carenza dei fattori del sistema del complemento, necessari per la rapida rimozione dei derivati cellulari.

La presenza di autoanticorpi anti-dsDNA, cioè quelli diretti contro il DNA nativo, è stata spesso fortemente associata al LES. Essendo considerati specifici per il LES, gli anticorpi anti-dsDNA sono stati inclusi nei criteri di classificazione della malattia. Tali anticorpi rappresentano un biomarker importante utilizzato per diagnosticare il LES. Inoltre, la presenza di anticorpi anti-dsDNA è spesso considerata fattore prognostico negativo nei pazienti con LES, a causa dell´elevato rischio di un coinvolgimento renale.

Secondo le attuali conoscenze, gli anticorpi anti-dsDNA sono direttamente coinvolti nella patogenesi della nefrite e della dermatite lupica, nonché in alcuni aspetti relativi all´interessamento del sistema nervoso centrale. Come gli anticorpi anti-dsDNA influenzino le restanti componenti cliniche incluse tra i criteri di classificazione attuali resta ancora da determinare.

Tuttavia, se si vuole dimostrare che gli anticorpi anti-dsDNA possano fungere da biomarker di riferimento nel LES, è importante tenere presente che essi non rappresentano una popolazione omogenea di anticorpi. Gli anticorpi che legano il dsDNA possono essere prodotti attraverso meccanismi diversi. Inoltre, è anche da chiarire se e come questi anticorpi siano patogeni. Alcuni individui possono produrre anticorpi anti-DNA a doppia elica senza mai mostrare segni clinici di nefrite o altro sintomo cardine del LES.

Gli anticorpi anti-DNA a doppia elica sono coinvolti anche nel fenomeno cosiddetto delle cellule del lupus eritematoso (LE), insieme con anti-istone e altri autoanticorpi e in presenza di attivazione della via classica del sistema del complemento. Tale fenomeno rappresenta il primo esame di laboratorio specifico in pazienti con LES, rilevata al microscopio ottico nel 1948 in preparazioni di midollo osseo. Indagini successive hanno dimostrato che il fenomeno è dovuto a leucociti polimorfonucleati (PMN) che fagocitano materiale cellulare necrotico, probabilmente derivato dall´alterato smaltimento di cellule apoptotiche presente in pazienti affetti da LES.

Nonostante la sua inclusione tra i disturbi immunologici elencati nei criteri ACR per la classificazione del LES, il fenomeno delle cellule LE non è attualmente utilizzato come esame di laboratorio di routine. Né le cellule LE, né qualsiasi altra singola analisi di laboratorio possono essere considerate come gold standard per

fare diagnosi di LES. La continua ricerca di nuovi potenziali biomarker si basa sulla moderna interpretazione della patogenesi della malattia.

Oltre al ruolo svolto nel fenomeno delle cellule LE, i PMN neutrofili rappresentano una delle cellule più interessanti coinvolte nella patogenesi del LES. La disfunzione delle trappole extracellulari dei neutrofili (NET) è stata recentemente dimostrata come un´alterazione che frequentemente colpisce pazienti affetti da LES. Circa il 40% di tutto il citoplasma e il 60% delle proteine totali nel citoplasma dei neutrofili è costituito da S100A8/A9, chiamata anche calprotectina. È una proteina il cui ruolo diagnostico è conosciuto soprattutto nelle malattie infiammatorie croniche intestinali. Tuttavia, nei primi anni novanta sono stati pubblicati i dati relativi al riscontro di un livello accresciuto di calprotectina nel siero dei pazienti con LES. Il ruolo della calprotectina nel LES non è stato ancora studiato a fondo. Nonostante la crescente evidenza di un possibile ruolo patogenetico che la calprotectina potrebbe svolgere nel LES, il suo dosaggio non è mai stato introdotto tra le indagini ordinarie o di follow-up della malattia.

Analogo discorso vale per l´Osteopontina. Si tratta di una proteina con molteplici funzioni, che inizialmente è stata scoperta a livello osseo, dove funge da ponte tra le cellule e la matrice minerale ossea. Lavori precedenti hanno segnalato che elevati livelli plasmatici di Osteopontina sono associati a determinate manifestazioni cliniche del LES. Tuttavia, l´utilizzo dell´Osteopontina nella diagnostica di laboratorio del LES non è ancora stato validato.

Obiettivo di questo progetto di ricerca è stato quello di fornire un contributo relativo alla valutazione di tradizionali e innovativi biomarkers nei pazienti affetti da malattie reumatiche, in particolar modo da LES.

Nella prima parte del progetto, l´attenzione-è stata focalizzata sul valore diagnostico e prognostico degli anticorpi anti-DNA a doppia elica in pazienti con recente esordio di sintomi reumatologici. La misurazione multipla e contemporanea degli anticorpi in 3 diversi laboratori ha messo in evidenza una notevole discrepanza di risultati, anche in caso di utilizzo del medesimo kit commerciale. Pazienti affetti da molte malattie, reumatiche e non reumatiche, sono risultati positivi al test per gli anticorpi anti-DNA, evidenziandone la bassa sensibilitá.

Tali anticorpi sono risultati inoltre essere significativamente associati alla presenza, nello stesso gruppo di pazienti, di particolari manifestazioni cliniche, quali nefrite, pleurite e coinvolgimento ematologico, il tutto a prescindere dalla diagnosi clinica.

Nella seconda parte della ricerca, su una popolazione ben selezionata di pazienti affetti da LES è stata effettuato un periodico monitoraggio clinico e di laboratorio per circa due anni. I dati raccolti sono stati analizzati al fine di individuare

eventuali correlazioni esistenti tra la presenza di tipiche manifestazioni cliniche del LES e la positivitá dei nuovi potenziali biomarkers indagati. Lo studio ha evidenziato che il fenomeno delle cellule LE, misurate con citometria di flusso, si associa alla contemporanea presenza di manifestazioni ematologiche e alla comparsa di manifestazioni cutanee nell´arco di circa due mesi. La contemporanea positivitá di altri biomarkers tradizionali, soprattutto la riduzione dei fattori del complemento, determina un aumento del rischio di contemporanea o imminente nefrite lupica.

L´analoga analisi finalizzata a valutare il ruolo di calprotectina e Osteopontina, nella stessa popolazione di pazienti affetti da LES, ha evidenziato significative associazioni con altrettanto importanti manifestazioni cliniche, quali nefrite, artrite e attivitá generale di malattia.

Tali risultati incoraggiano lo studio ulteriore di tali biomarkers in una piú ampia popolazione di pazienti, preferibilmente tramite studio multicentrico, nel tentativo di validarli all´uso clinico di routine. Qualora i nostri risultati venissero confermati, si aprirebbero nuovi scenari nel trattamento dei pazienti affetti da LES. La possibilitá concreta di prevedere l´insorgenza di importanti manifestazioni cliniche potrebbe infatti indurre ad un approccio terapeutico finalizzato alla prevenzione delle stesse, con probabile beneficio ai fini prognostici.

In attesa della verifica dei suddetti biomarkers o dell´introduzione di altri biomarkers affidabili, continueremo a utilizzare gli esami di laboratorio al momento disponibili soltanto come strumento di supporto nell´iter diagnostico, affidando la responsabilitá di una corretta diagnosi soprattutto all´acume clinico del medico e alla sua capacitá di mettere assieme tutti i vari tasselli che compongono il puzzle del LES.

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Appendix