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**BIOMARKERS
AND
MEDIATORS
IN
SYSTEMIC LUPUS
ERYTHEMATOSUS**

IFN α versus the CRP response, and evaluation
of suPAR and anti-dsDNA antibody assays

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ABSTRACT

Systemic lupus erythematosus (SLE) is a heterogeneous autoimmune disease which may affect multiple organ systems. Interferon alpha (IFN α) and autoantibodies that form immune complexes with nuclear antigens (ANA) are hallmarks believed to drive the disease into a vicious circle of inflammation, tissue damage, autoantigen exposure and autoantibody production.

In SLE, the disease course is characterized by episodes of exacerbations alternating with remissions. In order to best treat the patient it is important to closely monitor symptoms and signs of disease activity. Because of the disease heterogeneity, no single biomarker has yet been found to reflect SLE disease activity in general, although anti-double stranded DNA (anti-dsDNA) antibodies sometimes indicate activity, primarily with renal involvement, and constitutes an item of the SLE disease activity score SLEDAI-2K. However, the method of anti-dsDNA measurement is not standardized and therefore varies between different laboratories. In many other inflammatory conditions, such as rheumatoid arthritis and during bacterial infections, the C-reactive protein (CRP) level is a good indicator of ongoing inflammation, but in SLE and during viral infections, CRP commonly fails to reflect the degree of inflammation. Both viral infections and SLE are characterized by IFN α , and we thus aimed to elucidate whether IFN α can inhibit CRP production. Further, four assays for anti-dsDNA antibody measurements were evaluated with regard to SLE disease specificity and activity, and a new potential biomarker of inflammation, the soluble urokinase plasminogen activator receptor (suPAR), was assessed in relation to disease activity and organ damage.

An *in vitro* inhibitory effect of IFN α on CRP transcription and production was found in hepatocytes, and this was consolidated by *in vivo* studies of CRP and IFN α in sera from well-characterized SLE patients (KLURING; Kliniskt lupusregister i nordöstra Götaland). Here, CRP and disease activity were associated among patients without IFN α and without a CRP lowering gene variant (SNP rs1205). The poor disease activity compliance of CRP could therefore be explained, at least in part, by polymorphisms in the CRP gene and increased levels of IFN α . Critical differences between the methods measuring anti-dsDNA were found regarding disease specificity and ability to reflect disease activity and the results suggests the *Crithidia luciliae* immunofluorescence test (CLIFT) for diagnostic purposes and a bead-based multiplex assay (FIDIS) for monitoring of disease activity. Evaluation of suPAR in SLE revealed no association of suPAR with disease activity, but interestingly instead with accumulated organ damage. suPAR could therefore possibly be used to advert patients at high risk of organ damage.

A detailed biological and clinical characterization of established and emerging SLE biomarkers is of importance since it may improve the clinical management as well as increase the knowledge about disease mechanisms.

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SVENSK SAMMANFATTNING

Kroppens immunförsvar har som uppgift att försvara oss mot sjukdomsalstrande inkräktare (patogener) såsom bakterier och virus. För att immunförsvaret ska kunna identifiera dessa patogener krävs att det kan skilja på kroppsegna och kroppsfrämmande strukturer. Om denna mekanism fallerar kan det leda till autoimmunitet, vilket innebär att kroppens immunförsvar attackerar den egna vävnaden och i längden uppstår en kronisk inflammation.

Systemisk lupus erythematosus (SLE) är en autoimmun sjukdom som kan drabba många olika organ och som karaktäriseras av autoantikroppar mot strukturer som finns i cellkärnan, till exempel DNA. Antikroppar produceras av immunförsvarets celler och kan binda till patogener för att neutralisera och förstöra dessa. Vid SLE bildas (förutom vanliga antikroppar) även autoantikroppar och när dessa binder till strukturer i vävnaden orsakar de skada. Autoantikroppar mot dubbelsträngat DNA (dsDNA) är utmärkande för SLE och förekomst av dessa kan användas för att ställa diagnos.

Sjukdomsförloppet vid SLE kännetecknas av perioder med sjukdomsaktivitet som följs av lugnare perioder utan aktivitet och för att kunna behandla och motverka sjukdomsskov är det viktigt att följa graden av inflammation och patientens symptom noggrant. Vid många olika sjukdomstillstånd (exempelvis vid bakterieinfektioner) mäts mängden C-reaktivt protein (CRP, som ibland kallas snabbsänkan) i blodet för att fastställa graden av inflammation, men vid SLE stiger inte CRP trots kraftig inflammation. Detta gör att CRP inte återspeglar sjukdomsaktiviteten vid SLE.

I **Arbete I och II** har vi undersökt varför CRP inte reflekterar sjukdomsaktiviteten vid SLE. Resultaten visar att interferon alfa ($IFN\alpha$), en av immunförsvarets signalmolekyler som är förhöjd vid SLE, hämmar produktionen av CRP. En variant av CRP-genen verkar också kunna påverka CRP-nivåerna vid SLE. Vikten av att studera vilka faktorer som reglerar CRP-produktionen understryks av att CRP har en mängd biologiska funktioner som skulle kunna skydda mot SLE. Ett exempel är att CRP hjälper till att städa bort döda celler i kroppen. Om döda celler inte elimineras effektivt kan inflammation uppstå. Vid SLE har man både sett en ökad celldöd och en nedsatt förmåga att städa bort döda celler, och inflammationen som detta orsakar tros vara en central del i sjukdomsprocessen.

I **Arbete III** har vi utvärderat fyra olika metoder för att mäta autoantikroppar mot dsDNA. Många nya metoder för analys av dsDNA autoantikroppar har lanserats under senare år och

då dessa autoantikroppar är centrala i diagnostik såväl som övervakning av sjukdomsaktivitet är det av största vikt att mätmetoderna är ordentligt utvärderade. Vår studie visar att den äldre och etablerade metoden CLIFT fungerar bäst för diagnostik, medan FIDIS (en nyare metod) fungerar bättre för att följa sjukdomsaktivitet över tid.

I jakt på nya potentiella inflammationsmarkörer undersökte vi om nivåerna av suPAR (förkortning för löslig urokinas plasminogen aktivator receptor) återspeglar sjukdomsaktivitet vid SLE (**Arbete IV**). suPAR har nämligen visat sig fungera som en inflammationsmarkör vid många andra sjukdomstillstånd. Vi fann dock ingen association mellan suPAR och inflammation vid SLE. Däremot fann vi att mängden suPAR i blodet är associerad till mängden organskador som patienten ackumulerat under sin sjukdomstid och i fortsatta studier vill vi undersöka om suPAR kan förutspå och kanske till och med orsaka organskador.

Vidare studier av CRP och andra inflammationsmarkörer kan på sikt bidra till förbättrad behandling av SLE patienter och djupare insikt i sjukdomsmekanismerna.

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PAPERS IN THE THESIS

- I **Helena Enocsson**, Christopher Sjöwall, Thomas Skogh, Maija-Leena Eloranta, Lars Rönnblom, Jonas Wetterö. Interferon- α mediates suppression of C-reactive protein: Explanation for muted C-reactive protein response in lupus flares?
Arthritis & Rheumatism 2009;60:3755-3760
- II **Helena Enocsson**, Christopher Sjöwall, Alf Kastbom, Thomas Skogh, Maija-Leena Eloranta, Lars Rönnblom, Jonas Wetterö. Association of serum C-reactive protein levels with lupus disease activity in the absence of measurable interferon- α and a C-reactive protein gene variant.
Arthritis & Rheumatology 2014 (in press)
- III **Helena Enocsson**, Christopher Sjöwall, Lina Wirestam, Charlotte Dahle, Alf Kastbom, Johan Rönnelid, Jonas Wetterö, Thomas Skogh. Four anti-dsDNA antibody assays in relation to systemic lupus erythematosus disease specificity and activity.
Manuscript (submitted)
- IV **Helena Enocsson**, Jonas Wetterö, Thomas Skogh, Christopher Sjöwall. Soluble urokinase plasminogen receptor levels reflect organ damage in systemic lupus erythematosus.
Translational Research 2013;162:287-296

ABBREVIATIONS

ACR	American College of Rheumatology
ANA	Antinuclear antibodies
ANOVA	Analysis of variance
C/EBP β	CCAAT/enhancer binding protein β
C	Complement
CD	Cluster of differentiation
CLIFT	<i>Crithidia luciliae</i> immunofluorescence test
DMARDs	Disease-modifying antirheumatic drugs
CRP	C-reactive protein
DNA	Deoxyribonucleic acid
dsDNA	Double-stranded deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
Fc	Fragment crystallizable
FSGS	Focal segmental glomerulosclerosis
GWAS	Genome-wide association study
HLA	Human leukocyte antigen
Hep-G2	Hepatocellular carcinoma G2
IC	Immune complex
Ig	Immunoglobulin
IL	Interleukin
IL-1ra	Interleukin-1 receptor antagonist
IFN	Interferon
IFNAR	Interferon α/β receptor
IRF	Interferon regulatory factor
ITAM	Immunoreceptor tyrosine-based activation motif
LAP	Liver-enriched activator protein
LIP	Liver-enriched inhibitory protein
MMP	Matrix metalloproteinase
NETs	Neutrophil extracellular traps
NF- κ B	Nuclear factor κ B
PBMC	Peripheral blood mononuclear cells
PBS	Phosphate buffered saline
pDC	Plasmacytoid dendritic cell
PGA	Physician's global assessment

pSS	Primary Sjögren's syndrome
RNA	Ribonucleic acid
RA	Rheumatoid arthritis
SAP	Serum amyloid P component
SD	Standard deviation
SDI	SLICC/ACR damage index
SEM	Standard error of the mean
SLE	Systemic lupus erythematosus
SLEDAI-2K	Systemic lupus erythematosus disease activity index 2000
SLICC	Systemic Lupus International Collaborating Clinics
SNP	Single nucleotide polymorphism
snRNP	Small nuclear ribonucleoprotein
STAT	Signal transducer and activator of transcription
suPAR	Soluble urokinase plasminogen activator receptor
uPA	Urokinase-type plasminogen activator (also known as urokinase)
uPAR	Urokinase plasminogen activator receptor
TLR	Toll-like receptor

INTRODUCTION

Autoimmunity

The immune system protects the body from harmful microbes (pathogens) such as bacteria and viruses, but it can also cause disease itself if not being tightly regulated. Keys to the ability of the immune system to distinguish self (host structures) from non-self (pathogen structures) are the pattern recognition receptors and the deletion of potentially self-reactive lymphocytes. In autoimmune diseases, this ability has for some reason been challenged, resulting in loss of self-tolerance [1].

A textbook definition of autoimmune disease reads:

“A disease caused by a breakdown of self-tolerance such that the immune system responds to self antigens and mediates cell and tissue damage. Autoimmune diseases can be organ specific (e.g. thyroiditis or diabetes) or systemic (e.g. systemic lupus erythematosus)” [2]

Factors that may contribute to autoimmunity are heritability (preferably gene variants of the human leukocyte antigen; HLA), infections and toxins. The ability of infections to cause autoimmunity is attributed to the increased general inflammation that causes tissue destruction and activation of immune cells, which in a genetically predisposed individual can result in loss of self-tolerance. Another mechanism of infections to cause autoimmunity is the so called molecular mimicry. Some pathogen structures are similar to structures of the host and antibodies produced to eliminate the pathogen may hence cross-react with self structures. However, in most cases of autoimmunity a multitude of factors (both well-known and unknown) cooperates with coincidence to cause disease [1].

Systemic lupus erythematosus

Systemic lupus erythematosus (SLE) is a systemic autoimmune disease. The name lupus erythematosus refers to the facial skin rashes commonly found in SLE patients. It was the 13th century physician Rogerius that first used the term lupus (Greek for wolf) erythematosus (Latin for red) to describe these facial lesions resembling a wolf bite. However, the facial rash is only one of many signs and symptoms of SLE and as the disease was further characterized in the beginning of the 20th century, the word

“systemic” was added to describe the disseminated form of lupus erythematosus that can affect almost every organ of the body [3].

In SLE, there is a loss of self-tolerance which leads to autoantibody production, preferably antinuclear antibodies (ANA), immune complex (IC) formation, IFN α production, tissue inflammation, tissue destruction and organ dysfunction.

Clinical characteristics, classification criteria and diagnosis

SLE is an extremely heterogeneous disease, which constitutes a challenge to clinicians and patients, but also complicates research and drug development. Currently, there are no validated diagnostic criteria for SLE, and the diagnosis is therefore dependent on an experienced physician who can evaluate symptoms and laboratory findings. Patients with SLE commonly present with a variety of symptoms that, on their own, mostly are not unique to SLE. However, accumulation of manifestations and exclusion of other diagnoses may result in a clinical diagnosis. Further, there are available disease classification criteria which are often used to support a diagnosis, but the purpose of these criteria is rather to define the patient group in clinical trials and facilitate comparisons with other trials [4].

The first classification criteria were developed by the American College of Rheumatology (ACR) in 1971 [5]. These criteria were revised in 1982 (ACR-82) [6] and slightly modified (although not validated) in 1997 [7]. The ACR-82 criteria are shown in Table I and exemplify typical manifestations of SLE. Among the most common manifestations of SLE (>30% of patients) are arthritis/arthralgia, malar rash, fever and photosensitivity whereas epilepsy, psychosis, and oral ulcers constitute relatively rare manifestations [4,8]. In order to fulfil the ACR-82 criteria, at least four of the listed conditions must be met (Table I) [6]. Although these criteria (with or without the modification from 1997) have been widely used in clinical trials, the sensitivity for SLE was recently shown to be 86%, meaning that 14% of patients with true SLE fall outside these criteria [9]. In an attempt to increase the clinical relevance and to integrate new knowledge about SLE, new classification criteria were developed in 2012 by the Systemic Lupus International Collaborating Clinics (SLICC) group [9]. In these criteria, termed SLICC-12, the patient must fulfil 4 criteria as a minimum, of which at least one is clinical and one is immunological, *or* the patient must have a lupus nephritis, proven by biopsy, together with positive ANA and/or anti-double stranded DNA (anti-dsDNA) (Table II). SLICC-12 did result in a higher sensitivity (94%) and comparable specificity for SLE as compared to ACR-82 [9] and thus embraces a higher percentage of clinically

defined SLE patients. Although the SLICC-12 criteria would in theory perform better as a diagnostic tool, they are not evaluated for this purpose [9].

Long before the ACR-82 and the SLICC-12 criteria were developed, Fries and Holman tried to define the clinical praxis of diagnosis employed at many centers around the world [10]. These diagnostic principle was later referred to as the “Fries criteria” and comprise three different requirements; i) involvement of at least two typical organ systems, ii) presence of autoantibodies, and iii) exclusion of other diseases (Table III). Interestingly, this principle resemble the SLICC-12 criteria in that both clinical and immunological criteria must be fulfilled.

Table I. 1982 classification criteria for systemic lupus erythematosus*

Requirements: ≥ 4 criteria observed serially or simultaneously	
Criteria	Comments
1. Malar rash	Fixed erythema over the malar eminences
2. Discoid Lupus	Erythematous raised patches
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight
4. Oral ulcers	Oral or nasopharyngeal ulceration
5. Arthritis	Nonerosive arthritis involving 2 or more peripheral joints
6. Serositis	Pleuritis OR pericarditis
7. Renal disorder	Persistent proteinuria OR cellular casts
8. Neurological disorder	Seizures OR psychosis
9. Hematological disorder	Hemolytic anemia OR leukopenia (≥ 2 occasions) OR Thrombocytopenia
10. Immunological disorder	Positive LE cell preparation OR anti-dsDNA OR anti-Sm OR biologically false positive test for syphilis (Wassermann reaction)
11. Antinuclear antibody	ANA in absence of drugs associated with “drug induced lupus” syndrome

ANA = Antinuclear antibodies, dsDNA = double stranded DNA, LE = Lupus erythematosus

*Condensed from: Tan EM, Cohen AS, Fries JF, Masi AT, McShane DJ, Rothfield NF, et al. The 1982 revised criteria for the classification of systemic lupus erythematosus. *Arthritis Rheum* 1982;25:1271-7 [6].

Table II. SLICC classification criteria for systemic lupus erythematosus*

Requirements: ≥ 4 criteria (at least 1 clinical and 1 immunologic criteria) OR Biopsy-proven lupus nephritis with positive ANA or anti-dsDNA	
Clinical criteria	Immunological criteria
1. Acute cutaneous lupus	1. ANA
2. Chronic cutaneous lupus	2. Anti-dsDNA
3. Oral or nasal ulcers	3. Anti-Sm
4. Non-scarring alopecia	4. Antiphospholipid antibodies
5. Arthritis	5. Low complement (C3, C4, CH50)
6. Serositis	6. Direct Coomb's test (in the absence of hemolytic anemia)
7. Renal	
8. Neurological	
9. Hemolytic anemia	
10. Leukopenia	
11. Thrombocytopenia	

ANA = Anti nuclear antibodies, dsDNA = double stranded DNA, C3 = Complement component 3, C4 = Complement component 4, CH50 = 50% Haemolytic complement activity.

*Condensed from: Petri M, Orbai AM, Alarcon GS, Gordon C, Merrill JT, Fortin PR, Bruce IN, Isenberg D, Wallace DJ, Nived O et al: Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum* 2012, 64(8):2677-2686 [9].

Table III. Diagnostic principle for systemic lupus erythematosus according to Fries*

Requirements: Fulfillment of 1, 2 and 3	
1. Multisystem disease (≥ 2 systems)	
Skin:	Butterfly rash, oral ulcers, alopecia, diffuse skin rash, DLE
Joints:	Objective joint inflammation
Kidneys:	Glomerulitis, glomerulonephritis
Serositis:	Pleuritis, pericarditis, peritonitis
Blood:	Leukopenia, thrombocytopenia, hemolytic anemia
Lungs:	Transient infiltrates
Nervous system:	Seizures, psychosis, mononeuritis
2. Autoantibodies (at some time point, by any technique)	
3. Exclusion of other diseases (e.g. rheumatoid arthritis, scleroderma)	

*Condensed from: Fries JF, Holman HR: *Systemic lupus erythematosus: a clinical analysis. In: Major problems in internal medicine. Edited by LH S. Philadelphia, PA: W.B. Saunders; 1975: 8-20* [10].

Genetics and epidemiology

The prevalence of the disease ranges from 20 to 150 cases per 100,000 people with a great diversity of prevalence and disease manifestations between ethnic groups. As for most autoimmune diseases, females are more vulnerable to SLE (~90% of the SLE patients are females). The reason hereto is uncertain but may be due to estrogen or genes located on the X-chromosome [11].

Many genes or loci have consistently been linked to SLE, but the concordance for monozygotic twins is only 25%, implying that environmental factors are also necessary for development of the disease in genetically predisposed individuals [11]. Genome wide association studies (GWAS) in SLE patients have revealed a major genetic influence of the human leukocyte antigen (HLA) region (*e.g.* HLA-DR3 and HLA-DRB1) and genes related to B/T-cell signaling (*e.g.* protein phosphatase non-receptor type 22; PTPN22 and interleukin-10; IL-10). Further, genes involved in the complement cascade and type I interferon (IFN) pathways (reviewed in the Complement protein-, and IFN α sections, respectively) also predispose to SLE [12]. Most genes that associate with SLE are also involved in other autoimmune disease such as type I diabetes, rheumatoid arthritis (RA) and systemic sclerosis, indicating shared pathways of autoimmunity in general [12].

Pathogenesis of SLE and the waste disposal of dying cells

A malfunction in the elimination of dying cells is believed to play a central part in the pathogenesis of SLE and is commonly referred to as the defective waste disposal theory [13]. The normal turnover of cells in the human body demands an effective handling of dying cells, because otherwise these would accumulate and release harmful intracellular substances into the tissue. The normal procedure of waste disposal starts with the apoptotic cell which secretes “find-me” signals to recruit phagocytes, and then displays “eat-me” signals to facilitate recognition and ingestion of the dead cell (*i.e.* efferocytosis). The phagocyte then silently degrades the apoptotic cell and releases anti-inflammatory mediators [13,14]. Factors that regulates this process are for example the complement proteins [15], which recognize dying cells and opsonize them to enhance the phagocytosis [13], and the pentraxins (C-reactive protein; CRP, pentraxin 3; PTX3 and serum amyloid P; SAP) that binds to structures of late apoptotic or secondary necrotic cells and regulates phagocytic uptake and inflammation [16,17].

An increased apoptosis rate [18] as well as reduced phagocytic uptake of apoptotic material have been described in cells from SLE patients [19,20]. There are also several

other entries to disturb the waste disposal and to set off a vicious circle of accumulating dead cells, break-down of self-tolerance, autoantibody production, IC formation, tissue deposition, IFN α production and inflammation (Figure 1).

Complement proteins

A genetic defect of any of the early complement proteins of the classical activation pathway (C1q, C1s, C1r and C4) implies a high risk (>80%) of developing SLE [12,21]. Although such genetic complement deficiencies are extremely rare, they tell us about the importance of these proteins and the consequences of complement malfunction in the SLE pathogenesis. In fact, a majority of SLE patients have an acquired deficiency of complement proteins due to the consumption of complement proteins as the disease progresses with inflammation and tissue damage [21].

Autoantibodies

A defective and delayed clearance of apoptotic cells can result in secondary necrosis, implying a lost membrane integrity, release of danger signals and exposure of constituents from the cell nucleus [22]. A prolonged extracellular exposure of nuclear components can cause structural alterations which may result in immunogenic epitopes that are able to activate an immune response [13]. ANAs are examples of autoantibodies that are characteristic of SLE and include the anti-dsDNA, antibodies to U1 small nuclear ribonucleoproteins (snRNPs), anti-Smith (anti-Sm), anti-Ro/SSA and anti-La/SSB autoantibodies [8]. Interestingly, there are also studies showing an over-representation in SLE of autoantibodies against proteins that are involved in the elimination of cell debris, like C1q [23], serum amyloid P (SAP) [24], and CRP [25,26] (further described in the CRP section). Such autoantibodies may hinder the function of these proteins and thereby disturb the clearance of dying cells [27].

Figure 1. A selection of important and interrelated events in the pathogenesis of SLE.
 IFN α = Interferon alpha; pDC = Plasmacytoid dendritic cell; UV = Ultraviolet

Immune complexes and Fcγ receptors

Antibodies bound to their respective antigens are called immune complexes (ICs). In the context of an infection the antibodies neutralize toxins and pathogens and contribute to elimination via phagocytosis and activation of an appropriate immune response [1]. Due to the defective waste disposal in SLE, an excessive amount of apoptotic material may initiate an immune response with antigen presentation, subsequent T-cell activation and B-cell activation with production of autoantibodies (*e.g.* ANA). This abnormal production of autoantibodies may then result in IC formation [1,13]. Handling of ICs are to a great extent dependent on rapid classical complement activation with attachment of complement proteins (*e.g.* C3b) to the ICs. This makes them more soluble and less prone to aggregate and deposit in tissues [21,28]. A congenital complement deficiency, or deficiency due to exaggerated complement consumption thus further aggravates a scenario of accumulated ICs and dying cells. ICs can also be formed in the tissue (*in situ*) as exemplified by nucleosomes containing DNA and positively charged histones. Because the net charge of nucleosomes is positive, they tend to get trapped in the basement membrane of renal glomeruli which are negatively charged [29]. Anti-dsDNA antibodies may therefore bind nucleosomes in the kidney, activate complement and recruit immune cells that cause glomerulonephritis and in the long run also damage to the kidneys [30,31].

The ability of ICs to activate immune cells relies largely on the binding of constant regions (fragment crystallizable region; Fc) of the immunoglobulin G (IgG) antibodies to Fcγ receptors on the immune cells and by complement proteins binding to their receptors. Six different Fcγ receptors (FcγRs) are found in humans (FcγRI, FcγRIIa, FcγRIIb, FcγRIIc, FcγRIIIa and FcγRIIIb) of which all except the FcγRIIb have (or associates with) an activating domain called immunoreceptor tyrosine-based activating motif (ITAM) [2]. The subsequent actions upon IC binding to immune cells depends on the IgG subclass, the size of the IC, the type of immune cells and the array of FcγRs and complement receptors present on the cell surface, as well as the cytokine milieu [1,2].

An important route of IC-mediated inflammation in SLE is the activation of IFNα production via FcγRIIa-transportation of ICs to intracellular toll-like receptors (TLRs) [32]. This is further outlined below (IFNα section).

Neutrophil NETosis

A recently discovered type of cell death is the so-called NETosis of neutrophils [33]. This process is unique and distinct from apoptosis and necrosis. Activated neutrophils eject large amounts of chromatin (DNA and associated proteins) in a web-like structure

upon dying. These neutrophil extracellular traps (NETs) also contains LL-37, an antimicrobial peptide, and the NETs thus promotes both entrapment and killing of pathogens. In SLE, neutrophils are activated when they encounter ICs with nucleic acids and they can be further primed to NETosis by IFN α stimulation [34]. The NETosis will therefore nurture a feed forward loop of DNA release, IC formation, IFN α production and further DNA release. In addition, patients with anti-dsDNA antibodies are poor degraders of NETs. This is probably due to the binding of anti-dsDNA to NETs, which protects them from DNase I degradation [35]. Further, NETosis is associated with the release of reactive oxygen species, known to alter both DNA and proteins, making them more immunogenic [36].

Treatment

There is no cure for SLE but with appropriate medication, a majority of patients may stay in remission for long periods of time. Improvements in treatment and diagnostics over the years have prolonged the survival of SLE patients and today the 10-year survival rate is around 90% [37]. Although there is no such thing as an SLE-specific drug, but rather treatments for specific manifestations of the disease, glucocorticoids and antimalarial agents are frequently prescribed to SLE patients. Glucocorticoids hamper many immune functions and may cause harmful side effects at long term use. Disease modifying anti-rheumatic drugs (DMARDs) are therefore often used to minimize the need for glucocorticoids and to treat specific organ involvement [38], most notably the antimalarial agents (*e.g.* hydroxychloroquine), but also other immune-modulating drugs including antibodies targeting specific receptors (*e.g.* cyclosporine, azathioprine, mycophenylate mofetil, intravenous immunoglobulins and belimumab) [39,40]. Cyclophosphamide and anti-CD20 (rituximab) are commonly used to treat the most severe manifestations of lupus. Many of these drugs are not approved for SLE, but are commonly used when conventional therapy fails, since beneficial effects have been shown in uncontrolled trials [40].

Given the important role of IFN α in SLE, drugs targeting the IFN α pathways would appear to be a reasonable approach, yet no medicine with the primary aim to interfere with IFN α production or its actions is available. Of note, both antimalarials and glucocorticoids have been ascribed anti-IFN α effects [41,42], but it remains uncertain to what extent this mechanism contributes to the overall drug actions [43]. Drugs that are designed to specifically interact with IFN α pathways are currently under evaluation and some of them seem promising with regard to desirable effects as well as side effects [43,44].

Disease activity and organ damage in SLE

The SLE disease activity index 2000 (SLEDAI-2K) is a frequently used tool for the determination of disease activity in patients, and constitutes a range of clinical and laboratory findings (Table IV) [45]. Patients are considered to be in remission if the SLEDAI-2K score is zero whereas a score of 1-5 means a relatively low activity. A SLEDAI-2K score of 6-10 suggests moderate activity and a score above 11 indicates high activity. Further, an increase of SLEDAI-2K of more than 3 is considered to be a disease flare [46]. Continuous disease activity or relapsing-remitting disease increases the risk of patients to acquire permanent damage to organs which in turn increases mortality [47,48].

An instrument to assess organ damage of lupus patients was developed in 1996 [49]. This tool, named SLICC/ACR Damage Index (SDI) measures accumulated organ damage over time and may indicate disease severity and prognosis if interpreted together with SLEDAI-2K and overall health status of the patient [49]. Organ systems and examples of damages are indicated in Table V. SDI was created to include damages that are caused by the SLE disease activity itself (*e.g.* renal failure and skin scarring), or by medication side effects (*e.g.* cataract and osteoporosis) (Figure 2) [47]. However, any damage that fits into the SDI will be scored, irrespective of the cause. To avoid mix-up between ongoing disease activity and permanent organ damage, a SDI item has to be present for at least 6 months to be calculated [49].

A number of studies have confirmed the use of SDI as a prognostic tool and there is a strong association between SDI and mortality [48,50-52], particularly if damage occurs to the kidneys, and close to disease onset [53]. Early organ damage also predicts further damage (Figure 2) [47,48], and thus, a powerful treatment of patients with damage at or close to disease onset may be critical.

Table IV. SLEDAI-2K descriptors and scores*

SLEDAI-2K Score	Descriptor	Definition (short version)
8	Seizure	Recent onset, exclude metabolic, infectious or drug causes.
8	Psychosis	Altered ability to function in normal activity due to severe disturbance in the perception of reality.
8	Organic brain syndrome	Altered mental function.
8	Visual disturbance	Retinal changes of SLE.
8	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	Lupus headache	Severe, persistent headache; may be migrainous, but must be nonresponsive to narcotic analgesia.
8	Cerebrovascular accident	New onset of cerebrovascular accident(s). Exclude arteriosclerosis.
8	Vasculitis	Ulceration, gangrene, tender finger nodules, periungual infarction, splinter hemorrhages, or biopsy or angiogram proof.
4	Arthritis	≥ 2 joints with pain and signs of inflammation (i.e., tenderness, swelling or effusion).
4	Myositis	Proximal muscle aching/weakness, associated with elevated creatine phosphokinase/aldolase or electromyogram changes or a biopsy showing myositis.
4	Urinary casts	Heme-granular or red blood cell casts.
4	Hematuria	> 5 red blood cells/high power field. Exclude stone, infection or other cause.
4	Proteinuria	> 0.5 gram/24 hours
4	Pyuria	> 5 white blood cells/high power field. Exclude infection.
2	Rash	Inflammatory type rash.
2	Alopecia	Abnormal, patchy or diffuse loss of hair.
2	Mucosal ulcers	Oral or nasal ulcerations.
2	Pleurisy	Pleuritic chest pain with pleural rub or effusion, or pleural thickening.
2	Pericarditis	Pericardial pain with at least 1 of the following: rub, effusion, or electrocardiogram or echocardiogram confirmation.
2	Low complement	Decrease in CH50, C3, or C4 below the lower limit of normal for testing laboratory.
2	Increased DNA binding	Increased DNA binding by Farr assay above normal range for testing laboratory.
1	Fever	> 38°C. Exclude infectious cause.
1	Thrombocytopenia	< 100,000 platelets / $\times 10^9/L$, exclude drug causes.
1	Leukopenia	< 3,000 white blood cells / $\times 10^9/L$, exclude drug causes.

Abbreviations: SLEDAI-2K = SLE disease activity index 2000, C3 = Complement component 3, C4 = Complement component 4, CH50 = 50% hemolytic complement activity.

*Condensed from Gladman DD, Ibanez D, Urowitz MB (2002) Systemic lupus erythematosus disease activity index 2000. *J Rheumatol* 29: 288-91 [45].

Table V. A condensed version of the SLICC/ACR Damage Index (SDI)*

Organ system/item	Example(s) of common items	Maximum score
Ocular	Cataract	2
Neuropsychiatric	Epilepsy, cerebrovascular accident	6
Renal	Glomerular filtration rate < 50%	3
Pulmonary	Pulmonary infarction	5
Cardiovascular	Myocardial infarction, valvular disease	6
Peripheral vascular	Venous thrombosis	5
Gastrointestinal	Infarction or resection of bowel (below duodenum), spleen, liver or gall bladder	6
Musculoskeletal	Deforming erosive arthritis, osteoporosis with fracture	6
Skin	Scarring chronic alopecia	2
Premature gonadal failure		1
Diabetes		1
Malignancy		2

*Condensed from Gladman D, Ginzler E, Goldsmith C, et al. (1996) The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. *Arthritis Rheum* 39: 363-9 [49].

Figure 2. Causes and consequences of organ damage in SLE. Modified from Doria A, Gatto M, Zen M, Iaccarino L, Punzi L (2014) Optimizing outcome in SLE: treating-to-target and definition of treatment goals. *Autoimmun Rev*. In press [47].

Mediators and biomarkers in SLE

Due to the various manifestations of lupus and the great diversity in disease phenotype between patients, it has proven difficult to find biomarkers that mirror general inflammation (and/or organ damage) in SLE. The inflammatory activity in SLE is instead assessed based on an array of laboratory findings and symptoms, *e.g.* SLEDAI-2K or a global estimation of the disease activity assessed by an experienced doctor (*e.g.* the physician's global assessment; PGA) [54]. Despite validated indexes such as the SLEDAI-2K and SDI, it may sometimes be difficult to distinguish ongoing inflammation from symptoms/findings originating from permanent organ damage. A more straightforward way to assess disease activity as well as distinguish it from organ damage would thus increase the clinical management of SLE patients. The section below describes established as well as new mediators and biomarkers that were considered in this thesis and which are of relevance in SLE.

CRP

C-reactive protein (CRP) was first discovered by Tillett and Francis in 1930 [55] who found that a non-protein fraction of *Pneumococcus* bacteria (designated fraction C) precipitated with serum from patients in the acute phase of pneumonia infection, but also with serum from febrile patients with other diseases. Later it was discovered that it was the polysaccharide in the C fraction (C polysaccharide) extracted from *Pneumococci* that reacted with serum from acutely ill patients [56]. Avery with colleagues found that the serum reactant was a Ca^{2+} dependent protein [57], and designated it by its current name; CRP [58]. Since then, the research about CRP has revealed important knowledge about its structure, regulation and biological functions.

CRP is a positive acute phase protein, meaning that its concentration increases profoundly during inflammation. Together with pentraxin 3 (PTX3) and serum amyloid P component (SAP), CRP belongs to the pentraxin family of proteins [59]. As the name suggests, it is composed of five identical subunits (23.2 kDa each) and these are non-covalently linked in a cyclic formation [59-61]. The protein is structurally distinct on each side, having an effector face as well as a recognition face (Figure 3). The recognition face binds phosphocoline (PC) in a Ca^{2+} dependent manner, whereas the effector face has affinity for C1q and Fc γ Rs [60,62,63]. PC is found on many microbes but is also exposed on the host cell membrane during late apoptosis/necrosis [64]. By binding to PC on damaged cells or microbes via its recognition face, the effector face becomes available to C1q or Fc γ R binding, resulting in complement activation via the

classical pathway and/or phagocytosis of the dying cell or microbe. This configuration makes CRP an important participant in the waste disposal of dying cells and removal of pathogens [13,65].

Figure 3. The two faces of C-reactive protein, binding to distinct structures. The recognition face binds to surfaces and molecules to be taken care of, *e.g.* dying cells and pathogens whereas the other face mediates effector functions such as complement activation and phagocytosis.

CRP and its protective role in SLE

CRP has been ascribed preventive and disease-modifying properties in SLE due to a range of biological functions that are described below and illustrated in Figure 4.

When CRP is involved in complement activation, the complement cascade is more likely to be limited to the initial stages, including opsonization by C3b but excluding amplification by the alternative pathway of complement activation and formation of C5 convertase, thereby limiting an inflammatory response and preventing the formation of membrane attack complex (MAC) [66]. This is due to the ability of CRP to bind factor H [66,67], a complement regulatory protein that can displace Bb in the C3 or C5 convertase and consequently hamper further complement activation. Factor H also aids the actions of factor I, a serine protease that cleaves C3b and C4b, and thus further prevents C5 convertase formation [1]. Factor H remains active when bound to CRP, and CRP may therefore concentrate the complement regulatory actions of factor H to

surfaces decorated with CRP and prevent excessive inflammation at sites of tissue damage [67].

Apart from its ability to bind to and promote an anti-inflammatory removal of dying cells, CRP binds to nuclear components like U1 snRNP and histones [68-70] that are targets for antibodies in SLE. This can possibly facilitate the removal of these components, as well as hide autoantibody epitopes, which otherwise could be recognized by autoantibodies or immune cells. Further, extracellular histones might cause damage themselves by binding to cell membranes and induce increase of intracellular calcium, resulting in endothelial permeability, platelet aggregation and thrombocytopenia [71,72]. Recently, it was demonstrated that CRP, by its association with histones neutralizes these cell toxic effects [73].

Another feature of CRP that could be of importance in SLE is its ability to inhibit IFN α production from plasmacytoid dendritic cells (pDCs) that have been stimulated with snRNP/dsDNA containing ICs [74]. Because CRP can bind to, not only snRNP itself, but also Fc γ RIIa [62,63], it was investigated whether the inhibitory effect of CRP on IC-induced IFN α production was due to its blocking of snRNP uptake via Fc γ RIIa. This was, however, not the case. Instead, CRP was shown to alter the processing of ICs, leading to accelerated localization of ICs in late endosomal compartments [74], which may result in a diminished capacity to stimulate IFN α production [75]. As a side note, we recently carried out experiments similar to these, sometimes showing an inhibitory effect of CRP on snRNP-IC induced IFN α production from monocyte-depleted peripheral blood mononuclear cells (PBMCs), but sometimes this effect was not observed (not published).

In vitro experiments, carried out in murine models of SLE have confirmed the role of CRP as a mediator of SLE protection [76-80], and genetic associations between CRP and SLE (described in the CRP genetics section below) further suggest its importance in SLE.

Figure 4. Biological events in the pathogenesis of SLE and the potential role of CRP (red pentamer) as a break in the vicious circle of cell death, autoantigen exposure, autoantibody production, immune complex formation and tissue deposition, IFN α production and inflammation.

CRP synthesis

Hepatocytes are the main producers of CRP. Although other sites of CRP synthesis have been described [81-85], few studies have been replicated by others. The CRP synthesis is mainly regulated at the transcriptional level involving IL-6 and IL-1 β [86,87]. Due to restricted accessibility, and difficulties in the *in vitro* maintenance of primary hepatocytes at the laboratory, most studies of CRP transcription and synthesis have been performed in hepatocyte cell lines such as Hep3B, Hep-G2 and PLC/PRF/5 [88-90]. Cell lines generally require both IL-1 β and IL-6 to induce CRP production, whereas primary hepatocytes may induce CRP synthesis without IL-1 β [86,87,91]. The reason for this is unknown but indicates a significant difference between hepatic cell lines and primary hepatocytes. Transcription factors involved in IL-6 induced CRP transcription are the signal inducer and activator of transcription 3 (STAT3) and the CCAAT/enhancer binding protein β (C/EBP β), whereas IL-1 β induced CRP transcription is mediated by NF- κ B [86-88,92].

CRP as a marker of inflammation

Properties of CRP that makes it widely used as a marker of ongoing inflammation is its rapid increase and short half-life (19h), and the fact that its elimination rate is principally unaffected by diseases [93]. The concentration of CRP can increase up to 10,000 fold within hours of inflammation, with a peak around 48 hours. High CRP levels are found in patients with burns, bacterial infections and some inflammatory diseases such as RA [65]. On the contrary, viral infections [94] and disease exacerbations of SLE [95-98] are generally not mirrored by a CRP response that is indicative of the ongoing inflammation, despite increased levels of IL-6 [96]. Because of its suggested role as an inhibitor of pathogenic events in SLE, it would be of importance to find the mechanism behind this phenomenon. Autoantibodies to C-reactive protein, which are overrepresented among SLE patients [26,99] and correlate with disease activity [25,100,101], have not been found to correlate with CRP levels [99,102]. Such antibodies are also shown to target modified (*e.g.* monomeric) CRP, and not the native (pentameric) structure [25,26,101] and would thus not constitute an explanation to low CRP in SLE. Further, a study undertaken to examine the elimination rate of CRP could not demonstrate an altered elimination rate in SLE patients compared to healthy controls or other disease states [93]. Altogether this implies an altered transcriptional control of CRP in SLE rather than an increased clearance of CRP. Since SLE and viral infections are characterized by a relatively low CRP response, but also by increased levels of IFN α (further described in the IFN α section), we intended to explore a possible role of IFN α as a suppressor of the CRP response. This was investigated in **Paper I** and **Paper II**.

CRP genetics and SLE

The CRP gene is located on chromosome 1 at position q21-q23. Several studies have shown polymorphisms of the *CRP* gene that affect baseline CRP levels as well as CRP levels during the acute phase response [103-105]. There are also studies showing a relation between SLE and genotypes/haplotypes of CRP that are associated with lower CRP levels [105,106]. Genetically low CRP could therefore constitute an explanation of low CRP in patients with SLE, but since patients normally mount an appropriate CRP response during bacterial infections [107,108], the genetic influence should be accompanied by other explanations to fully describe the causes of low CRP in SLE. rs1205 is a well-studied polymorphism of the CRP gene of which the rare allele is associated with low CRP concentrations and SLE predisposition [106,109]. This polymorphism (G > A) locates to the 3' untranslated region of the gene and was investigated in relation to CRP-levels in **Paper II**. Except from a few rare genetic variations in the CRP gene, all polymorphisms are either in the untranslated region, or so-called synonymous polymorphisms (*i.e.* no exchange in amino acid) in the exons [103,110], indicating a conserved and thus important biologic role of the protein.

IFN α

IFN α belongs to the type I IFNs comprising many IFN types, of which IFN α and IFN β are considered most important in humans [111]. IFN α further includes 12 subtypes with a sequence homology of 75-99% [111]. All type I IFNs signal through the same receptor; the IFN α/β receptor (IFNAR), which is present on all cells of the body and consist of two subunits: IFNAR1 and IFNAR2 [111]. Binding of type I IFNs to IFNAR (Figure 5) generally results in formation of a complex consisting of STAT1, STAT2 and interferon regulatory factor 9 (IRF9), collectively called interferon-stimulated gene factor 3 (ISGF3). The translocation of ISGF3 to the nucleus activates transcription of interferon-stimulated genes via binding to interferon stimulated response elements (ISRE). Synthesized proteins then mediate the antiviral, antiproliferative and immunomodulating effects of IFN α [112,113].

Although both subunits of IFNAR are required for IFN α binding, the nature of IFN α -receptor interaction and downstream effects seems to be dependent on the IFN α subtype [112]. This was elegantly described by Jaks *et al.* who evaluated 7 type I IFNs with respect to IFNAR affinity and kinetics, as well as the antiproliferative and antiviral activity of different IFNs [112]. The IFNAR2 domain was shown to bind all type I IFNs with higher affinity than IFNAR1, and there was a correlation between IFNAR2 affinity and ISGF3 activation (antiviral effects). Of the investigated IFN α subtypes (IFN α 1, 2, 8 and 21), IFN α 8 and IFN α 2 showed the highest affinity for IFNAR2, whereas IFN α 1 had relatively high IFNAR1 affinity and therefore stronger antiproliferative effects [112]. Alternative dimer formations of STAT (Figure 5, pathways to the right) can possibly explain the various effects of IFN α -IFNAR interactions [113,114]. The overall biological outcome of IFN α signaling may therefore be determined by cell type, STAT expression levels/patterns and the specific mixture of IFN α subtypes present in the environment [113].

IFN α in SLE

Although most cells can secrete small amounts of IFN α , the far most efficient IFN α producers are pDCs [115]. These cells express the endosomal toll-like receptor (TLR) 7 and TLR9, and respond to viral nucleic acids with massive production of IFN α . Generally, TLR7 and TLR9 encounter viral nucleic acids when the cell is infected by viruses but in SLE, these receptors may be activated by endogenous DNA or RNA (*e.g.* found in nucleosomes and snRNP) [116,117]. Circulating self-nucleic acids are normally non-immunogenic and physically separated from the endosomal TLRs, but when complexed with IgG, they can be transported to endosomes via Fc γ RIIa (Figure 6) [32]. This activates interferon regulatory factors (IRFs) and NF κ B leading to production

of IFN α and other proinflammatory cytokines [118]. Recently, the antibacterial peptide LL37, and the high mobility group box 1 (HMGB1), both of which are released during cell death, were shown to facilitate IC induced IFN α production [119,120]. Cell debris and ICs containing nucleic acids may therefore serve as endogenous IFN α inducers in SLE. The biological consequences of IFN α that have been implicated in the pathogenesis of lupus have recently been reviewed in detail [121] and include the facilitation of antigen presentation via increased upregulation of class I and II major histocompatibility complex (MHC), maturation of dendritic cells and stimulation of B cells to become antibody producing plasma cells [122,123]. Although far from all SLE patients have measurable IFN α in serum [124,125], the majority present with increased expression of type I IFN regulated genes, the “type I IFN signature” [126].

Figure 5. Signaling pathways activated by type I interferons (IFNs). Binding IFN α results in recruitment of signal transducer and activator of transcription (STAT) proteins. The three predominant STAT complexes that are formed control distinct gene-expression programs. The interferon-stimulated gene factor 3 (ISGF3) complex (which is composed of STAT1, STAT2 and IFN-regulatory factor 9 (IRF9)) binds to IFN-stimulated response element (ISRE) sequences to activate antiviral genes, whereas STAT1 homodimers bind to gamma-activated sequences (GASs) to induce pro-inflammatory genes. STAT3 homodimerization repress inflammatory pathways but the details remain obscure. *Reprinted by permission from Macmillan Publishers Ltd: Nature Reviews Immunology, Ivashkiv LB et al. Copyright 2014 [113].*

The significance of IFN α in SLE pathogenesis is also demonstrated by the fact that medical treatment with IFN α , *e.g.* IFN α 2b (Intron®A) in cancer and viral infections, may induce autoantibody production and in rare cases lupus like disease, *i.e.* drug induced lupus [127,128]. Serum levels of IFN α (and/or a presence of type I IFN signature) also correlate with SLE disease activity, and are associated with presence of anti-dsDNA antibodies, nephritis, symptoms from the central nervous system, and hematological manifestations [124,125,129,130]. In addition, GWAS of SLE has revealed a major influence of single nucleotide polymorphisms (SNPs) of IFN α -related genes, such as IRF5, IRF7, IRF8 and STAT4 [12].

Figure 6. IFN α production in SLE. Immune complexes that contain nucleic acids (*e.g.* snRNP and nucleosomes) are internalized to endosomes via Fc γ receptor IIa (FcγRIIa). The subsequent interaction of endosomal toll-like receptors (TLRs) with nucleic acids results in production of IFN α and other proinflammatory cytokines by interferon regulatory factor 9 (IRF9) and nuclear factor kappa B (NFκB) activation, respectively.

Anti-dsDNA

Anti-dsDNA autoantibodies were discovered in sera from SLE patients as early as in 1957 [131]. These autoantibodies were later found to be valuable for the diagnosis of SLE as they are rarely found in sera of healthy controls or patients with other autoimmune diseases (apart from autoimmune hepatitis), but are found in many SLE patients at least sometime during their disease course [132,133]. However, the reported frequencies of anti-dsDNA positive SLE patients is highly varying and seems dependent on method of anti-dsDNA assessment and the disease severity of the patients [133]. A pathogenic role of anti-dsDNA antibodies of IgG class has been suggested (Figure 7), primarily based on findings of dsDNA-anti-dsDNA deposits in kidneys of patients with lupus nephritis [134], and induction of nephritis-like disease in animal models by introduction of anti-dsDNA antibodies [30,135]. Another way for anti-dsDNA antibodies to cause kidney disease may be via cross-reaction with epitopes found in the kidney glomeruli such as α actinin [30,136] or with C1q [137] which can deposit in kidneys and whose autoantibodies are associated with lupus nephritis [138,139]. The source of anti-dsDNA containing ICs found in glomeruli could be circulating ICs that are trapped in the kidneys, but it is also suggested that ICs can be formed *in situ*, either by cross-reacting anti-dsDNA antibodies or by the entrapment of nucleosomes in the glomeruli and subsequent binding of anti-dsDNA/anti-histone antibodies [30,31,140]. A direct transportation of anti-dsDNA and other ANAs into the nuclei of living cells, known as *in vivo* ANA, has also been demonstrated [135,141], but it remains uncertain whether this mechanism is clinically relevant. It has also been suggested that this could simply be an artefact of *in vitro* tissue fixation [142].

Numerous assays for the measurement of anti-dsDNA are available on the market today, but the method for anti-dsDNA assessment is not specified in the ACR-82 criteria or SLICC-12 criteria [6,9]. The only anti-dsDNA method validated for the purpose of assessing disease activity by SLEDAI-2K is the Farr assay [45]. However, in clinical practice, this assay is nowadays rarely used due to included radioactive compounds and the availability of other more straightforward assays [133]. Today, the most commonly used techniques for the measurement of IgG anti-dsDNA are the *Crithidia luciliae* immunofluorescence test (CLIFT) and enzyme-linked immunosorbent assays (ELISAs) [133]. CLIFT detects anti-dsDNA antibodies by their binding to circular dsDNA, provided as the kinetoplast of *Crithidia luciliae*. The kinetoplast is a large network of concentrated circular dsDNA inside of a giant mitochondrion which is found in close proximity to the flagellum of *Crithidia luciliae* [143]. More recent assays for the analysis of anti-dsDNA, *e.g.* ELISA and bead-based assays, often use purified recombinant circular dsDNA as a target. Such dsDNA is not in a natural context (*i.e.*

bound to histones), and may incorporate a risk of detecting antibodies that are irrelevant from a clinical perspective [144].

Figure 7. Mechanism of anti-dsDNA formation and pathogenicity in SLE as suggested by Manson and Isenberg [30]. *In vivo* ANAs are autoantibodies that can enter and bind to nuclear structures of living cells. Ab = antibodies. ANA = antinuclear antibodies

suPAR

The cellular receptor for urokinase-type plasminogen (uPAR; CD87) is a multi-ligand receptor expressed on various cell types, which is involved in processes like proteolysis, cell migration and cell adhesion [145,146]. uPAR belongs to the lymphocyte antigen 6 (Ly-6) superfamily of proteins and is composed of three domains designated D1, D2 and D3 (Figure 8), of which D3 attaches to the cell membrane via a glycosyl phosphatidylinositol anchor (GPI). The molecular weight of uPAR ranges from 35 kDa to 60 kDa depending on the glycosylation pattern [147].

Biological implications of uPAR and suPAR

The complex functions and collaborations of uPAR and its ligands have recently been reviewed by Smith and Marshall [146] and is briefly described below. Interaction of uPAR with one of its ligands, urokinase-type plasminogen activator (uPA, also known urokinase) occurs primarily at the D1 domain [148], and results in the cleavage of plasminogen to plasmin, a protease involved in fibrinolysis and tissue remodeling. uPAR also interacts with integrins, speculatively via the D2 and D3 domains, and it is believed that uPAR cooperates with integrins to coordinate adhesion to extracellular matrix (ECM) and cell migration [146]. A third ligand for uPAR is vitronectin [149], an ECM protein that can bridge uPAR with $\beta 3$ integrins via its dual binding domains. Such bridging can mediate a close and strong contact of integrins to the extracellular matrix, and represents one of many coordinated effects suggested for uPAR and its ligands [146].

Expression of uPAR has been demonstrated on immune cells such as monocytes [150], activated T-cells [151], activated natural killer cells and neutrophils [152], but also on tumor cells, megakaryocytes [153], endothelial cells [154] and smooth muscle cells [155]. Its expression is mainly regulated by various growth factors [156-158] and formation of soluble uPAR (suPAR) is mediated via shedding of uPAR from the cell surface. However, an alternative splicing of uPAR messenger RNA resulting in uPAR without a GPI-anchoring site has also been demonstrated [159]. Cleavage sites of uPAR are present at the GPI-anchor and between the D1 and D2 units, resulting in three possible soluble forms (full length D1-D3, D2D3 and D1) and two membrane bound configurations of uPAR (full length and D2D3) (Figure 8). Enzymes that have been demonstrated to cleave uPAR are uPA itself, [160], plasmin [160,161], matrix metalloproteinases (MMPs) [162], GPI-specific phospholipase D [163] neutrophil elastase and cathepsin G [164], but other proteases are most likely involved. Release of suPAR has been demonstrated upon cell-cell contact [165,166], stimulation with proinflammatory cytokines [151,167], lipopolysaccharide (LPS) [166] and growth

factors [167]. The biological functions of the different suPAR forms are not entirely clarified, particularly not for D1 which is only found in urine [145]. The D2D3 fragment has chemotactic properties due to a SRSRY motif in the linker region between the D1 and D2 domains that is exposed upon cleavage. However, non-chemotactic D2D3 forms can also be generated if cleavage occurs within the SRSRY region [168]. Studies on chemotactic D2D3 have demonstrated involvement in stem cell mobilization [169] and leukocyte trafficking, effects that were mediated by the binding of suPAR to formyl peptide receptor (FPR), FPR-like 1 (FPRL1) and FPRL2 [152,170,171]. Full length suPAR seems to retain uPA binding as well as vitronectin binding properties [172,173], but unlike uPA-dependent cleavage of uPAR, uPA cannot cleave the soluble form [174]. Thus, suPAR could possibly keep uPA and vitronectin away from cell bound uPAR and thereby inhibit uPA-uPAR-vitronectin mediated proteolysis, cell adhesion and migration [145].

Figure 8. Surface-bound and soluble fragments of urokinase plasminogen activator receptor (uPAR). uPAR is expressed on various cell types and enzymatic cleavage of uPAR and suPAR results in various fragments with known and unknown functions. MMP = matrix metalloproteinase, uPA = urokinase plasminogen activator,

suPAR - a supermarker of inflammation?

Increased uPAR expression and circulating levels of suPAR were initially described to associate with tumor progression and prognosis in various forms of cancer [145,175]. Later, uPAR was found to be up-regulated on monocytes and lymphocytes in HIV infected individuals and serum suPAR was shown to predict survival independently of

established prognostic markers of HIV like CD4⁺ cell count and viral load [145,176]. Until today, suPAR has been investigated as a biomarker for inflammation and survival in various infectious and inflammatory diseases including tuberculosis, malaria, sepsis, cardiovascular disease and RA [145,152,177,178], and is claimed to reflect overall immune activation and systemic inflammation [145]. There is also a study demonstrating increased suPAR levels among SLE patients with high disease activity [179].

Recently, suPAR caught attention in the common kidney disorder focal segmental glomerulosclerosis (FSGS). This disorder is characterized by damage to podocytes, cells that are crucial for glomerular integrity, and results in loss of plasma proteins into the urine [180]. In cases where genetic defects of the podocytes are absent, no other known cause of the disease has been described [180], until recently when Wei *et al.* suggested suPAR to be a causative factor of FSGS [181]. A majority of FSGS patients had increased levels of suPAR, and via its activation of β 3 integrins on podocytes, suPAR was shown to mediate effacement of podocyte protrusions [181]. A central role of suPAR in FSGS has however been questioned by others, showing no differences in suPAR levels between FSGS and other kidney diseases [182].

Serum levels of suPAR are dependent on age and sex [183], but they seem relatively insensitive to diurnal variations [184]. Further, a high *in vitro* stability of suPAR in serum/plasma over time and freeze-thaw cycles has been demonstrated [185], which increases the interest of suPAR as a biomarker.

AIMS

The aim of this thesis was to unravel the mystery of the low CRP levels observed during SLE flares and to evaluate established and new biomarkers in SLE. Specific aims are listed below:

- To test the hypothesis of an IFN α dependent inhibition of CRP by *in vitro* studies (**Paper I**) and *in vivo* studies of SLE patient sera (**Paper II**).
- To examine the clinical utility of anti-dsDNA measurements by different assays with regard to SLE specificity and disease activity (**Paper III**).
- To evaluate the use of suPAR as a biomarker of disease activity and/or organ damage in SLE (**Paper IV**).

METHODOLOGICAL CONSIDERATIONS

CRP

Animal models for CRP studies

It would be desirable to study CRP regulation *in vivo* in animal models in order to confirm results from cultured cells. However, the classic pentraxins found in humans, CRP and SAP, differ in expression between mice and humans. In humans, CRP is an acute phase protein, meaning that it increases during inflammation, whereas SAP is constitutively expressed. The opposite is found in mice where SAP is the acute phase protein while CRP levels remains low during inflammation [186]. Rat CRP differs structurally from human CRP [187-189] and is not able to activate complement [189]. Mice and rats are therefore inappropriate animal models for studies of the human CRP response. In contrast, rabbits have a CRP regulation similar to humans [190]. The protein shares 70% strict amino acid identity with human CRP [191], have similar biological functions [192], and respond to inflammation with a significant increase [190,193]. Hence rabbit could perhaps be a suitable animal model for *in vivo* experiments of CRP synthesis.

In vitro analysis of CRP transcription and synthesis

In vitro studies of CRP synthesis are challenging due to the limited availability of CRP producing cells that are suitable for *in vitro* culturing. Although several cell types have been reported to produce CRP [83,85,194], human primary hepatocytes are indeed the most suitable model, but the cost is reflected by the poor availability of liver tissue and the short *ex vivo* lifespan of these cells in culture. In addition, most commercial hepatocyte cell lines with a retained CRP synthesis, e.g. Hep3B and PLC/PRF/5, are infected with hepatitis B [195] and are therefore less tempting to handle at the lab. Hep-G2 is a non-infected hepatocellular carcinoma cell line that is widely used and easy to culture, however, it lacks an endogenous CRP synthesis. In **Paper I**, HepG2 cells transfected with the CRP-promoter and a reporter gene (luciferase) was used. These cells were stably transfected with the 1-kb CRP promoter (HepG2-ABEK14 cells) and were kindly provided by Dr Jan Torzewski *et al.* [90]. Although this cell line performed well, its readout is not the actual synthesis of CRP, but instead CRP promoter activation. Hence, confirmatory studies were performed in primary human hepatocytes (Clonetics® Ready Heps) that were isolated, seeded on collagen coated plates and freshly shipped from Lonza (Rockland, ME, US).

CRP in cell culture

CRP is a sensitive molecule requiring Ca^{2+} and physiological pH to maintain its pentameric structure and ability to bind PC [59]. In previous studies of the biological effects of CRP, recombinant CRP preparations were accused of being contaminated by endotoxin or the cell toxic preservative sodium azide [196,197]. Further, it was speculated that the integrity of CRP could be lost in certain preparations, resulting in different ratios of native (pentameric) versus modified (monomeric) CRP, and that monomeric CRP would have other properties than the native form [198]. Although most of these complaints were questioned by others [199-201], later studies of CRP primarily use human isolated CRP that has been dialyzed in order to remove preservatives such as sodium azide. CRP used for PBMC stimulation at our lab (CRP from pooled human plasma, Sigma Saint Louis, MO, US) was dialyzed by the use of a dialysis cassette (γ irradiated Slide-A-Lyzer™, 10K MWCO from Thermo Scientific, Rockford, IL, US). PBS supplemented with Ca^{2+} was used as dialysis buffer and the last dialysis step was performed in cell culture medium.

Anti-dsDNA assays

The four anti-dsDNA assays evaluated in **Paper III** all detect serum IgG autoantibodies to dsDNA, but are otherwise different. The source of antigen, the solid phase of antigen attachment, the detection antibody and the detection principle differs between methods and can therefore affect the results. A schematic illustration describing the methodology of these assays is given in Figure 9, and further details can be found in the manuscript (**Paper III**). An important aspect is the ability of the methods to quantify anti-dsDNA. EliA and FIDIS are true quantitative methods, whereas CLIFT is semi-quantitative and EUROLINE is a qualitative assay according to the manufacturer, although the results are presented in a quantitative fashion (densitometry). In CLIFT, samples are evaluated visually from 2-step serial dilutions and therefore only gives information on how much the serum can be diluted without loss of signal. Practically this means that the difference in anti-dsDNA levels between two titers ranges from > 0 to a 4-fold increase. FIDIS and EUROLINE have the advantage of allowing detection of many different autoantibodies at the same time. The kit used for anti-dsDNA detection by FIDIS is called “FIDIS Connective Profile” and detects 14 different autoantibodies which are associated with connective tissue. The EUROLINE kit (ANA Profile 5) detects autoantibodies to 18 different antigens. Detection of other autoantibodies than those directed against dsDNA was however not considered when evaluating the assays.

Figure 9. Principles of detection in the four anti-dsDNA assays evaluated in **Paper III**. The readout of all assays but EUROLINE is fluorescence (colored arrows), either visually interpreted (CLIFT) or detected by a sensor (FIDIS and ELiA). CLIFT and FIDIS have fluorophore-conjugated detection antibodies (black) whereas ELiA uses an enzyme-conjugated detection antibody converting a substrate into fluorescence. The detection antibodies in EUROLINE are conjugated with an enzyme converting its substrate into a colored product. As solid support for dsDNA, ELiA uses polystyrene wells, EUROLINE has dsDNA coated membranes, and FIDIS uses dsDNA coupled to polystyrene beads. In CLIFT, the dsDNA is found in the kinetoplast of *Crithidia luciliae*. Yellow antibodies represent the anti-dsDNA in patient sera. β -Gal = β -galactosidase; AP = Alkaline phosphatase; FITC = Fluorescein-isothio-cyanate; PE = Phycoerythrin.

suPAR ELISA

The ELISA used to measure suPAR (ViroGates, Copenhagen, Denmark) detects the D1-D3 and D2D3 fragments and is verified for detection of suPAR in plasma. Since only serum was available from the patients included in KLURING, we performed a correlation study on plasma and serum collected simultaneously from 7 individuals with different kinds of infections. The results demonstrated a high correlation ($r = 0.993$, $p < 0.0001$) between serum suPAR and plasma suPAR (Figure 10). Thus measurement of suPAR in serum was considered valid.

Figure 10. Pearson correlation of plasma and serum soluble urokinase plasminogen activator receptor (suPAR) collected simultaneously in 7 individuals and measured by enzyme-linked immunosorbent assay (ELISA). r = Pearson correlation coefficient.

SLE patients

KLURING

KLURING is a Swedish acronym for Clinical lupus register in the northeast Gothia (Swedish: Kliniskt LUPusRegister i Nordöstra Götaland). Inclusion criteria in KLURING were a “clinical” SLE diagnosis, informed consent and fulfilment of at least 4 of the 11 ACR-82 classification criteria (Table I) [6] and/or fulfilment of the Fries criteria (Table III) [10]. Further, patients should be able to fully understand the details and consequences of participation. In 2012, the SLICC group established new SLE classification criteria (SLICC-12) (Table II) [9], resulting in 179 (88%) KLURING patients satisfying these new standardized SLE classification criteria. Details about number of patients fulfilling different criteria sets are shown in Table VI.

Table VI. Fulfillment of different SLE criteria among KLURING patients (n=198)

Criteria	This/all these criteria N (%)	Any of these criteria N (%)	Only this/these criteria N (%)
ACR-82	160 (81)	N/A	3 (2)
Fries	195 (98)	N/A	14 (7)
SLICC-12	179 (90)	N/A	0 (0)*
ACR-82 and Fries	157 (79)	198 (100)*	3 (2)
ACR-82 and SLICC-12	155 (78)	184 (93)	1 (1)
Fries and SLICC-12	178 (90)	178 (90)	3 (2)
ACR-82, SLICC-12 and Fries	154 (78)	198 (100)	N/A

*Inclusion in KLURING requires fulfillment of the ACR-82 and/or the Fries criteria.

ACR = American college of rheumatology; SLICC = Systemic lupus international collaborating clinics.

Serum samples from the SLE patients were collected at study inclusion and at every visit to the rheumatology clinic thereafter, meaning different timespans between visits for each individual. For consecutive analyses, 18 patients were selected based on fluctuating disease activity over time with a SLEDAI-2K score of at least 4 at some time point. Further, they were chosen to represent different disease manifestations and to comprise patients with detectable IFN α levels at inclusion (72%). Number of visits ranged from 2 to 14. For every consecutive patient, the visit with the highest and lowest disease activity, respectively, was determined based on SLEDAI-2K and PGA, with PGA as the primary determinant. If patients presented with the same disease activity at two or more visits, the first visit (chronologically) was chosen.

Serum samples

Collected serum samples were stored at -70°C and thereafter thawed and divided into aliquots. High sensitivity CRP (hsCRP) analyses and multiplex bead analyses were performed directly after the first thawing, whereas other cytokines and molecules were analyzed from aliquots that had been freeze-thawed 2-3 times. However, specific analyses of the different analytes were performed in aliquots originating from the same freeze-thaw cycle to minimize differences due to variation in sample handling [202]. An exception was the analysis of anti-dsDNA antibodies where sera had been freeze-thawed several times, with no guarantee that they were from the same freeze-thaw cycle. Anti-dsDNA antibodies are, however, relatively stable and show negligible variation in detectability over freeze-thaw cycles and storage time in EliA as well as CLIFT (Charlotte Dahle, Linköping University Hospital, personal communication).

Patient selection (Paper II-IV)

The patient selection is somewhat different for the three papers involving KLURING patients. In **Paper II**, we were limited to IL-6 analyses, which was only performed in sera from patients fulfilling the ACR-82 criteria (n=162). Further, the diagnostic principle according to Fries is not always accepted for publication purposes. After corrections in the database regarding fulfilled ACR criteria, we ended up with 155 patients fulfilling the ACR-82 criteria and with IL-6 data available.

During the writing of **Paper III**, the SLICC-12 classification criteria were introduced and we thus selected patients fulfilling the ACR-82 and/or the SLICC-12 criteria (n=184). Serum samples for analysis of anti-dsDNA by all four methods were available for 178 of these patients.

Paper IV was written during 2012 when the SLICC-12 criteria had not yet been introduced and all KLURING patients were therefore selected for this study. However, data are presented both with and without the patients fulfilling only the Fries criteria to clarify the result and to facilitate comparisons with other patient materials.

RESULTS AND DISCUSSION

IFN α and CRP in SLE (Paper I & II)

The lack of distinct correlation of CRP levels with disease activity in SLE [95-98] has long been a mystery. Our hypothesis, suggesting that low levels of CRP in SLE disease flares could be a result of high levels of IFN α , originated from the fact that both SLE and viral infections present with increased IFN α and/or an IFN α signature [130,203], as well as relatively low serum CRP. This hypothesis was investigated both *in vitro* (**Paper I**) and *in vivo* (**Paper II**) (Figure 11).

Figure 11. Schematic illustration of the IL-1 β /IL-6 induced C-reactive protein (CRP) production during inflammation, and its inhibition by interferon alpha (IFN α) which is commonly increased in SLE and viral infections. IL = Interleukin.

Hepatic CRP promoter activity and CRP production is attenuated by IFN α

We found an inhibiting effect of IFN α on IL-1 β /IL-6-induced CRP promoter activity in a transfected hepatocyte cell-line. 12 subtypes of IFN α were tested and all showed a different degree of inhibition, ranging from 25% to 47% with the strongest inhibitory effect by IFN α subtype 10. Two mixtures of IFN α subtypes were also tested, with the purpose of resembling the *in vivo* pattern of IFN α (Figure 12A). Purified IFN α isolated from virus-stimulated leukocytes (Purified leukocyte IFN; PLIFN) hampered the CRP response by 56%, whereas IFN α from snRNP-IC stimulated pDCs had an inhibitory effect of 49% (Figure 12A). Confirmatory studies in IL-6/IL-1 β stimulated primary hepatocytes revealed a comparable effect of IFN α (Figure 13). The suppressive effect was dependent on the receptor for IFN α / β (IFNAR) (Figure 12B). Importantly, neither the number of viable cells, nor the transferrin production was affected by IFN α , indicating a specific reduction in CRP promoter activity that was not due to a general decrease in cell viability/proliferation or protein synthesis.

Figure 12. The inhibitory effect of interferon alpha (IFN α) in IL-1 β plus IL-6 stimulated HepG2-ABEK14 cells. A) Inhibition of C-reactive protein (CRP) promoter activity by purified leukocyte IFN α (PLIFN), supernatants from plasmacytoid dendritic cells (pDCs) exposed to small nuclear ribonucleoprotein (snRNP) immune complexes (pDC-sup), or IFN α 2b (Intron®A). B) Absence of IFN α 2b-dependent inhibition in cells treated with an antibody that inhibits the IFN α / β receptor (IFNAR). Values are depicted as mean $\pm$ SEM from 3 independent experiments. *** = $p < 0.001$ versus unstimulated cells (by one-way ANOVA with Dunnett's post-test), ** = $p < 0.01$ versus isotype control (by t-test).

Figure 13. Effects of IFN α 2b on C-reactive protein (CRP) secretion by primary human hepatocytes. CRP secretion was induced by IL-1 β , IL-6, or IL-1 β plus IL-6. Values are the mean $\pm$ SEM from 2-3 independent experiments.

The mechanism of IFN α -dependent inhibition of CRP is unknown

As a continuation of this work we searched for the detailed molecular mechanism of the inhibition, but despite great efforts we could not identify the pathways of IFN α -dependent CRP inhibition (unpublished results). STAT1, which is activated by IFN α , has the ability to down-regulate STAT3 [204], a mediator of CRP transcription [87,205], and a possible shift in the phosphorylation between STAT1 and STAT3 due to IFN α was therefore investigated by a bead-based multiplex assay. To begin with, results indicated that STAT3 phosphorylation was hampered by IFN α . However, the manufacturing of the kit was delayed and we had to try another method (ELISA) by which we were not able to confirm our previous results.

In another approach, the relative amount of the short and long form of the transcription factor C/EBP β was investigated. Liver-enriched activating protein (LAP) is the active and full length form of C/EBP β whereas liver-enriched inhibitory protein (LIP) is a short form of the protein that serves as an antagonist of LAP [206]. We hypothesized that IFN α would increase the amount of LIP and thereby inhibit the effect of LAP, but this could not be verified. Further, secretion of IL-1 receptor antagonist (IL-1ra) and IL-6 was measured in hepatocyte cultures to study whether IFN α increases the IL-1ra production or reduces the IL-6 synthesis, and thereby decreases the effect of IL-1 β or IL-6 on CRP transcription, but no such effect was found.

CRP reflects lupus activity in the absence of IFN α and a CRP gene variant

SLE patients in the KLURING cohort were used to study the possible inverse relationship between IFN α and CRP *in vivo*, as well as the impact of a well-studied CRP polymorphism, rs1205, where the minor (rare) allele is associated with low CRP levels [105]. Serum samples from 155 KLURING patients, all meeting the ACR-82 criteria, were utilized. SLE patients exhibited higher CRP concentrations than a healthy control population but, as could be expected, there were no differences in CRP levels between SLE patients with high versus low disease activity (Figure 14).

Figure 14. Comparisons of serum C-reactive protein (CRP) levels in healthy controls and systemic lupus erythematosus (SLE) patients (Mann-Whitney test). SLE patients had significantly higher levels of CRP compared to healthy controls (A), but there was no significant difference in CRP levels between patients with active disease (SLE disease activity index, SLEDAI ≥ 6) and patients with low/no disease activity (SLEDAI < 6) (B). Bars represent median values.

A total of 36 patients (23%) had detectable levels of IFN α by a dissociation-enhanced lanthanide fluorescent immunoassay; DELFIA (detecting all IFN α subtypes except for subtype 8). 82 patients (53%) had one or two minor alleles of rs1205. To study the impact of these two potentially CRP-lowering factors, we compared the association of CRP and disease activity (SLEDAI-2K) in different subsets of the patients. We also studied the association between CRP and IL-6 in these patient groups (Table VII). A regression analysis, adjusting for age and body mass index (BMI), with SLEDAI-2K or IL-6 as response variables clearly

showed a lack of association between SLEDAI-2K and CRP levels, as well as between IL-6 and CRP levels among all patients. However, examining patients who did not have detectable IFN α , there was an association between IL-6 and CRP. When limiting the patient group to those having two major (common) alleles of rs1205 there was an association between SLEDAI-2K and CRP levels as well as IL-6 and CRP levels. Finally, examining patients with neither detectable IFN α , nor the rs1205 minor allele, an even stronger association was found (Table VII). This indicates an important impact of IFN α and CRP genetics on the ability of CRP to become increased in response to inflammation.

Table VII. Association of SLEDAI-2K and IL-6 levels with logCRP levels

Patient selection	n	SLEDAI-2K		IL-6	
		<i>p-value</i>	<i>Beta</i>	<i>p-value</i>	<i>Beta</i>
1. All patients	155	0.2		0.9	
2. IFN α <1U/mL	119	0.1		0.001	0.29
3. rs1205 major alleles*	73	0.005	0.33	0.001	0.37
4. Combination of 2 and 3	57	<0.0005	0.52	0.001	0.41

*Homozygous for the major allele (G). P-values and standardized beta coefficients (Beta) are from a multiple linear regression analysis adjusting for age and body mass index. CRP = C-reactive protein, SLEDAI-2K = Systemic Lupus Erythematosus Disease Activity Index 2000, IFN α = Interferon alpha, IL-6 = Interleukin 6.

CRP in patients followed over time

Serum samples from the 18 KLURING patients, selected for consecutive analysis, were used to evaluate the ability of CRP to reflect disease activity at an individual level. As shown in Figure 15, there was no significant difference in CRP level between the visit with the lowest and the highest disease activity ($p = 0.074$) as determined by Wilcoxon's matched signed rank test. Among these 18 patients, 3 had non-detectable IFN α levels and 8 had at least one minor allele of rs1205. Only one patient had non-detectable IFN α together with the major alleles of rs1205. Interestingly, this patient was found among the 3 patients having a CRP increase exceeding the CRP cut-off used in clinical routine (10 mg/L) (Figure 15). Further, this patient had the most dramatic increase (74 fold) in CRP concentration when comparing the lowest and the highest disease activity (red circles in Figure 15). This is thus an atypical SLE patient with high disease activity (SLEDAI = 7, PGA = 2), no known infection, and a CRP response indicating inflammation. Although no conclusions can be drawn from one individual case, it

clearly exemplifies that CRP may mirror SLE disease activity in the absence of IFN α and the minor allele of rs1205.

Figure 15. CRP levels at lowest and highest disease activity determined in 18 SLE patients that were followed consecutively. Red dots indicate the patient who had undetectable IFN α levels and no minor allele of rs1205. Dashed line indicate cut-off for CRP measurements in most "non-high sensitivity" assays. Highest and lowest disease activity is based on SLE disease activity index 2000 (SLEDAI-2K) and Physician's global assessment (PGA) scores.

Discussion about CRP and IFN α

Given the plausible role of CRP in waste disposal and protection from autoimmunity, it is important to understand the regulation of its production, as well as to elucidate the mechanisms by which it exerts its effects. CRP properties that are likely to interfere with the pathogenesis of SLE are i) the binding to dying cells and to nuclear constituents [207], ii) modulation of cytokine production [76], and iii) its inhibition of IFN α production [74] (Figure 4). In support of this, favorable effects of CRP have been demonstrated in animal models of SLE and/or nephritis [76-79]. However, a study trying to replicate these *in vivo* results failed [208]. Further, the inhibitory effect of CRP on snRNP-IC induced IFN α production [74] demonstrated by Mold *et al.*, could not be confirmed by us when performing similar experiments. Discrepancies among studies could possibly indicate differences in CRP preparations, but there are no obvious differences explaining the diverse results. Functional studies of CRP showing a beneficial role in autoimmunity or SLE have been performed by a

limited number of research groups and thus, a growing body of independent confirmatory studies are highly warranted to elucidate the role of CRP in SLE.

Based on the inhibitory effect of IFN α on CRP, clinical trials evaluating novel treatment options for SLE patients should take into account that a modest increase in CRP not necessarily means a disease exacerbation in these patients. Instead, it could actually indicate a reduction in IFN α levels and subsequent normalization of CRP values. Reduced IFN α levels, as a suggestion, could thus have dual beneficial effects, both due to reduction of harmful effects of IFN α , but also for allowing an appropriate CRP response. On the other hand, drugs inhibiting the actions of IL-6 (*e.g.* Tozilizumab) which are used to treat RA, could hypothetically worsen SLE disease course due to inhibition of CRP-production.

Future studies on CRP and IFN α may include a long-time follow-up in a selection of patients categorized either as non-CRP responders (detectable IFN α and minor allele of rs1205) or as CRP-responders (no detectable IFN α and major alleles of rs1205). Another study design could be to investigate CRP levels in rabbits subjected to inflammation and IFN α . In addition, the intracellular signaling of IFN α responsible for CRP suppression needs further investigation. Here, the differences in CRP-suppressive capacity among different IFN α subtypes (Figure 1B in **Paper I**) could give a clue to which STATs that are activated [112,209]. Stimulation of hepatocytes with other type I IFN, such as IFN β could perhaps also give information about signaling pathways involved in the suppression of CRP.

To conclude, our results indicate an attenuating effect of IFN α on the CRP response. Together with CRP gene polymorphisms, this provides an explanation to the absence of correlation between CRP and disease activity in SLE patients. Additional factors are however likely to contribute to the low CRP levels seen in SLE patients, and the role of CRP in SLE demands further investigation.

Clinical utility of different anti-dsDNA antibody assays (Paper III)

This study was undertaken to compare four assays for the determination of anti-dsDNA antibodies with regard to disease specificity and ability to reflect disease activity.

Using patients with RA, primary Sjögren's syndrome (pSS) and healthy individuals as references, the SLE specificity was highest for CLIFT (98%) and lowest for FIDIS (92%). The highest number of anti-dsDNA positive SLE patients was detected by EliA (sensitivity of 35%) and the lowest number by CLIFT (24%) (Figure 16). All methods correlated significantly with each other ($\rho \geq 0.41$) with the highest concordance (84%) demonstrated between FIDIS and CLIFT, and the lowest concordance between EliA and EUROLINE (72%). Concordance was calculated by dividing the sum of double-positive and double-negative patients with the total number of patients.

Figure 16. Levels of anti-dsDNA in SLE patients and control groups measured by FIDIS (A), EliA (B), CLIFT (C) and EUROLINE (D). Disease (SLE-) specificity of different anti-dsDNA antibody assays was calculated in relation to control groups; rheumatoid arthritis (RA), primary Sjögren's syndrome (pSS) and healthy controls (HC). The dashed line indicates cut-off for positivity. Percentages indicate proportions of positive individuals in each (disease) group.

FIDIS performed best regarding correlation with disease activity variables in cross-sectional samples (Table VIII), but also reflected disease activity (SLEDAI-2K and PGA) in consecutive samples with a higher accuracy compared to the other methods. Further, a comparison of anti-dsDNA levels between the visit with the highest and the lowest disease activity was performed in the patients followed over time. In this comparison, EliA and FIDIS had the highest number of patients who presented with increased anti-dsDNA levels (>25% or difference in titer) when disease activity increased.

Fisher's exact test revealed differences between the assays regarding associations of anti-dsDNA with certain disease manifestations (ACR-82 criteria). The most striking difference was shown for anti-dsDNA assessed by CLIFT, which was negatively associated with skin manifestations (ACR 1, 2 or 3). Further, anti-dsDNA measured by FIDIS was positively associated with hematologic disorder, in contrast to the other methods where no such association was found. In addition, a phenotype including renal disorder (ACR 7) was only significantly reflected by EliA and FIDIS but not by EUROLINE and CLIFT.

Table VIII. Spearman's correlation between disease variables and anti-dsDNA antibody levels assessed by four assays

Variables	FIDIS		EliA		CLIFT		EUROLINE	
	rho	p-value	rho	p-value	rho	p-value	rho	p-value
Classical complement function (n=169)	-0.552	<0.0005	-0.426	<0.0005	-0.333	<0.0005	-0.195	0.011
C4 (n=177)	-0.495	<0.0005	-0.362	<0.0005	-0.284	<0.0005	-0.209	0.005
C3 (n=178)	-0.371	<0.0005	-0.251	0.001	-0.218	0.003	-0.154	0.040
IFN α (n=178)	0.323	<0.0005	0.269	<0.0005	0.215	0.004	0.137	0.068
ESR (n=178)	0.193	0.010	0.176	0.019	0.112	0.135	0.081	0.280
SLEDAI-2K ^a (n=178)	0.148	0.048	0.121	0.109	0.096	0.203	0.034	0.657
PGA (n=178)	0.109	0.148	0.160	0.033	-0.001	0.984	0.012	0.876

^aItem for anti-dsDNA (by CLIFT) excluded. C = Complement fragment, IFN α = Interferon alpha, ESR = Erythrocyte sedimentation rate, SLEDAI-2K = Systemic Lupus Erythematosus Disease Activity Index 2000, CRP = C-reactive protein, PGA = Physician's global assessment

Discussion about anti-dsDNA assays

Among the four assays compared we recommend the use of CLIFT to determine IgG anti-dsDNA status (positive or negative), and FIDIS to monitor disease activity. An insensitivity of CLIFT to detect small differences in anti-dsDNA levels could probably account for its poor capacity to mirror disease activity. On the other hand, CLIFT was highly specific for SLE which could perhaps be explained by the natural conformation of the antigen (kinetoplast of *Critidia luciliae*), possibly capturing antibodies of high clinical relevance [144]. The low SLE specificity of FIDIS was not due to a high number of anti-dsDNA positive healthy controls, but rather to anti-dsDNA positive RA and pSS patients. This could speculatively indicate an interference of the antigen with other autoantibodies, since RA and pSS are both autoimmune diseases. This is further suggested by the fact that all anti-dsDNA positive RA patients, as assessed by FIDIS, also had autoantibodies recognizing IgG (rheumatoid factor; RF), and 9 out of 10 had autoantibodies to cyclic citrullinated peptides (anti-CCP). In contrast, only 2 out of 5 anti-dsDNA positive patients, as assessed by EliA, were RF positive, and 1 out of 5 was anti-CCP positive. It could be important to notice that SLE patients with erosive arthritis may have anti-CCP antibodies, but that there is a discrepancy between RA and SLE in that most “anti-CCP antibodies” found in SLE patients are citrulline independent, meaning that they also react with unmodified cyclic peptides (containing arginine) and therefore are not true anti-CCP antibodies [210]. Commercial assays commonly used for anti-CCP measurements (as was also the case in the present study) do not discriminate between true and false anti-CCP antibodies [210] and a possible cross-reaction between CCP and dsDNA may therefore be dependent on its nature (citrullinated or not). Although these thoughts are highly speculative, it justifies further investigation.

Discrepancies between assays in their ability to detect anti-dsDNA relevant for disease activity could be due to the nature of the antigen in the assay. The repertoire of anti-dsDNA autoantibodies in SLE patients is most likely diverse and dependent on the epitope spreading scheme of each individual [30]. Therefore, patients may react differently to the antigens with regard to antigen specificity and affinity. The source of antigen as well as its immobilization on the solid phase could contribute to differences in epitope exposure and thus capture a diverse range of autoantibodies. An example of this is the relationship between disease phenotype (fulfilled ACR criteria) and anti-dsDNA antibodies among assays demonstrated in this study. Renal involvement was reflected by anti-dsDNA assessed by EliA and FIDIS, but not by EUROLINE or CLIFT. Further, patients that are anti-dsDNA positive by CLIFT seem to be protected from skin involvement. An assay that specifically detects highly pathogenic anti-dsDNA antibodies would likely be the best method both for monitoring of activity and for diagnostic purposes.

Aspects that were not considered in the paper, but that could be of importance at the clinical laboratory are the amount of time and money consumed by the different assays. FIDIS and EUROLINE could possibly be regarded as advantageous, since many different autoantibodies are detected at the same time. On the other hand, such information is not always requested and may confuse the clinical interpretation of laboratory results. Recent studies comparing anti-dsDNA assays reveal an outstanding SLE specificity of CLIFT, but various results when it comes to sensitivity and ability to mirror or predict disease activity [133,211,212]. Further evaluation of anti-dsDNA methods may discern promising assays detecting anti-dsDNA of clinical importance. This may subsequently contribute to recommendations about applicable anti-dsDNA measurement techniques and cut-off limits in SLEDAI-2K and SLE classification criteria [213].

To monitor and predict organ damage in SLE (Paper IV)

suPAR as a biomarker of organ damage

The emerging biomarker of inflammation and mortality, suPAR, was measured in serum samples from KLURING patients with the hypothesis that it could be marker of disease activity in SLE. Although an association between SLE disease activity and suPAR levels has recently been reported by others [179], no such association could be demonstrated by the present study. Instead, suPAR was found to associate with organ damage as defined by SDI. There was a strong correlation ($r = 0.550$) between suPAR and SDI, but since suPAR levels are dependent on age and sex, we analyzed the association in a multiple regression analysis. SDI was strongly associated with suPAR both when investigating all patients in KLURING, and also when including only patients fulfilling the ACR-82 criteria (Table IX). To reveal which kind of damages had the greatest impact on suPAR levels, the SDI was divided into specific organ systems, and tested in a multiple linear regression model. The results from this model suggests a major impact of renal damage, and modest impacts of ocular -, neuropsychiatric -, skin -, and peripheral vascular damage on suPAR levels (Table IX). Only a few of the patients had SDI scores for some of these specific organs which imputes uncertainty (type I error) into the analysis. Among all patients ($n = 198$), 9 had damage to the kidneys, 15 had ocular damage, 8 had skin damage and 16 had peripheral vascular damage. Further, other organs might have failed to show an impact due to low numbers of patients (type II error). Another important aspect is the difference in maximum score for the respective organ systems. Neuropsychiatric organ damage has a maximum score of 6, whereas the maximum score for skin is 3, and 2 for ocular damage. This makes some organ systems less capable of influencing the regression model.

Table IX. The impact of organ damage (SDI) on suPAR levels

Variable	All patients				ACR-82 patients			
	Model 1*		Model 2†		Model 1*		Model 2†	
	$\beta^{\ddagger}$	p-value	$\beta^{\ddagger}$	p-value	$\beta^{\ddagger}$	p-value	$\beta^{\ddagger}$	p-value
Global SDI	0.50	<0.0005	0.48	<0.0005	0.45	<0.0005	0.43	<0.0005
Organ systems[§]								
Renal	0.34	<0.0005	0.31	<0.0005	0.34	<0.0005	0.31	<0.0005
Ocular	0.23	<0.0005	0.23	<0.0005	0.21	0.003	0.20	0.005
Neuropsychiatric	0.21	<0.0005	0.22	<0.0005	0.19	0.004	0.21	0.002
Skin	0.19	0.001	0.19	0.001	0.20	0.002	0.20	0.003
Peripheral vascular	0.14	0.019	0.13	0.023	0.15	0.020	0.13	0.026

*Adjusted for age and sex

†Adjusted for age, sex, leukocyte count, platelet count and prednisolone dose

‡Standardized beta coefficient (SD increase)

§Only organ systems that were retained in the model after a stepwise analysis are shown

SDI = Systemic Lupus International Collaborating Clinics/American College of Rheumatology Damage Index, suPAR = soluble urokinase plasminogen activator receptor, ACR-82 = 1982 American College of Rheumatology classification criteria

Can suPAR predict organ damage?

Findings from our suPAR study brought on discussions about the potential value of suPAR as a predictor of organ damage (SDI) in SLE. In support of this assumption, Eugen-Olsen *et al.* have demonstrated that elevated suPAR levels are associated with an increased risk of developing cancer, cardiovascular disease and type 2 diabetes in a general population [183]. The association was also shown independent of CRP, which is known to predict cardiovascular events [183,214]. To investigate the possible predictive value of suPAR in SLE, the suPAR levels at disease onset (SDI = 0) could be investigated in relation to SDI some years later (SDI $\geq$ 0). The KLURING cohort constitutes only a small number of patients with disease onset at study inclusion and thus an alternative approach was conducted to test the predictive value of suPAR. The change in SDI between study inclusion (2008-2011) and 2013 was calculated and associated with suPAR levels at inclusion in KLURING. For deceased patients, the last available SDI was used. Patients that moved to another rheumatology clinic before 2013 were excluded.

Data from this study (unpublished results) demonstrate significantly higher suPAR levels in patients with a SDI increase compared to patients without an SDI increase (Figure 17). There

was also a higher percentage of deceased patients in the groups of SDI increase. It is, however, known that organ damage may predict further organ damage in SLE [48], wherefore the suPAR levels might still reflect the organ damage at inclusion but not necessarily predict future increase in SDI. Among the KLURING patients, there was a weak correlation between SDI at inclusion and SDI increase over time ($\rho = 0.33$, 95% CI 0.20-0.45). To examine the impact of SDI at inclusion and suPAR, respectively, on SDI increase over time, a multiple regression analysis was performed with SDI at inclusion, suPAR and age as independent variables. Age had the highest association with SDI increase ($p < 0.0005$, standardized $\beta = 0.35$), whereas suPAR had a weaker association ($p = 0.005$, standardized $\beta = 0.20$). SDI at inclusion was not even retained in the stepwise analysis. From these results we conclude that suPAR may predict organ damage independently of SDI at inclusion, but that a study examining suPAR levels at the time of diagnosis would be preferable and reduce the biases.

Figure 17. Levels of suPAR in patients with different SDI increase. suPAR was measured at study inclusion and the SDI increase was calculated by subtracting the inclusion SDI from the SDI value of 2013 (resulting in an interval of 2-5 years). Kruskal-Wallis with Dunn's post test. Percentages indicate the proportion of deceased patients in each SDI category. SDI = SLICC Damage Index.

Discussion suPAR

As suPAR has been suggested not only to reflect inflammation, but also to contribute to organ damage [181], it is of interest to follow the ongoing debate regarding suPAR in FSGS [182,215]. Results from the initial study, suggesting suPAR as a causative agent of FSGS via $\beta 3$ integrin activation [181] has been questioned by later studies [215], and a number of limitations in the study design has been pointed out by Maas *et al.* in a recent article discussing suPAR in FSGS [215]. First of all, it is unclear which fragment of suPAR that may

be responsible for the damage in FSGS, and further, suPAR is inversely correlated to glomerular filtration rate (GFR, *i.e* kidney function) which was not adjusted for in the study. To explore a possible bias due to reduced kidney function in our study, urine albumin as well as creatinine was included in the regression models, but did not abolish the strong association between suPAR and SDI. In addition, when patients with renal damage were excluded from the analysis, there was still a significant association between suPAR and SDI. Further evaluation of the linkage between renal function and blood levels of suPAR are needed in order to clarify a potential mechanistic role in FSGS as well as its potential use as a biomarker in diseases where renal function may be impaired such as SLE.

In contrast to our study, Toldi *et al.* have demonstrated an association between SLE disease activity and suPAR levels [179]. Although there is no obvious explanation for the diverse results, their study had another design and did not adjust for age and sex, two factors known to influence suPAR levels [183].

The present work suggests a potential role of suPAR as a marker of organ damage in SLE, but does not explain the underlying mechanism. Many questions still remain to be answered regarding the biological functions of suPAR, and its role as a biomarker for inflammation and tissue damage. For instance, it is not known whether suPAR only mirrors uPAR activity, implying proteolysis and cell migration, or if it is actively secreted to contribute in these (and perhaps other) processes. Further, little is known about the relative amount of the different fragments of suPAR in biological fluids, and whether they reflect and/or contribute to inflammation and tissue damage differently. Altogether, the potential use of suPAR as a biomarker in SLE as well as in other diseases demands further clarifications.

CONCLUSIONS

The acute phase reactant CRP is widely used as a biomarker of inflammation, but it often fails to reflect disease activity in SLE. This thesis investigated the role of IFN α as a potential suppressor of CRP. Further, four anti-dsDNA assays were evaluated with regard to SLE specificity and disease activity, and an up-coming biomarker of immune activation, suPAR, was evaluated as a marker of disease activity and organ damage in SLE. The findings are concluded below:

- Hepatic CRP promoter activity and protein synthesis are inhibited by IFN α which could thus explain the lack of association with inflammation in SLE and perhaps also in viral infection. In support of this finding, we found CRP to associate with SLE disease activity among patients without measurable IFN α and without a genetic variant of the *CRP* gene associated with low levels of CRP.
- The four anti-dsDNA assays that were evaluated had a relatively low concordance and differed in specificity and sensitivity, as well as in the correlation of anti-dsDNA with disease activity measures. CLIFT had the highest specificity, and is thus suitable for diagnostic purposes. FIDIS showed the strongest correlation with disease activity variables, and is therefore more suitable for monitoring of disease activity.
- Although suPAR seems to reflect general inflammation in a wide range of infectious and inflammatory diseases, it did not associate with disease activity in SLE. Instead we found a strong association between suPAR and SLE organ damage (SDI). The potential of suPAR as a predictor of organ damage will be investigated in future studies.

An ultimate SLE biomarker would be a stable molecule that is easy to sample, cheap to measure and that discriminates between disease activity, infections and organ damage. Even though it is unlikely that such biomarker will be found, the search for new candidate biomarkers and evaluation of established biomarkers will hopefully bring new insights into SLE pathogenesis and management.

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