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Biomarkers of disease activity and organ damage in systemic lupus erythematosus

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Jag tror att när vi går igenom tiden

att allt det bästa inte hänt än

Håkan Hellström

ABSTRACT

Systemic lupus erythematosus (SLE) is a systemic inflammatory disease. Clinically, the distinction between ongoing inflammation attributed to SLE, and organ damage due to medication or co-morbidities remains challenging. In addition, SLE is a heterogeneous disease where the various disease phenotypes complicate the search for biomarkers that adequately reflect disease activity and/or signs of increasing organ damage. The aim of the thesis was to investigate and evaluate potential new biomarkers of disease activity and/or organ damage in SLE patients.

High mobility group box protein-1 (HMGB1) is a nuclear non-histone protein that can shuttle to the cytoplasm, become secreted extracellularly, and participate in systemic inflammation. Administration of monoclonal anti-HMGB1 antibodies has been reported both to attenuate and intensify disease in animal models of arthritis and lupus. In Paper I of the thesis, circulating anti-HMGB1 was found in 23% of the SLE patients and correlated with disease activity variables. The biological role of these autoantibodies remains to be elucidated.

As a consequence of massive circulating levels of cellular debris and immune complexes, SLE patients have insufficient capacity to remove such material via the reticuloendothelial system. Pentraxin 3 (PTX3) may possibly protect against lupus flares due to classical complement activation, opsonization of apoptotic cells, and cytokine induction. In Paper II, circulating PTX3 was found to be inhibited or exhausted by interferon (IFN)- α , a key cytokine of SLE pathogenesis, and serum levels of PTX3 in SLE patients were inversely related to IFN- α levels. Suppressed PTX3 levels may contribute to a vicious circle resulting in impaired waste clearance, autoantigen exposure and autoantibody production, and sustained disease activity.

Osteopontin (OPN), a protein known to influence cell signaling and apoptosis, has been proposed as a marker of organ damage in pediatric lupus. In a Swedish cross-sectional study, circulating OPN levels were found to be raised in SLE (Paper III). In patients with recent-onset disease, OPN reflected disease activity, while in established disease, OPN appeared to mirror damage accrual and cardiovascular damage. In Paper IV, OPN was instead analyzed in an international longitudinal multi-center study based on patients with recent-onset SLE and

follow-up data. OPN turned out to be a poor predictor of organ damage, but significant associations were observed between OPN and disease activity both at disease onset, as well as over 5 years of follow-up.

In conclusion, increased anti-HMGB1 antibody and decreased PTX3 levels could potentially sustain the impaired waste-disposal. Of the molecules analyzed in this thesis, OPN seems to be the best marker of disease activity. Further studies of these proteins may help to better understand SLE pathogenesis and to optimize treatment of patients.

POPULÄRVETENSKAPLIG SAMMANFATTNING

Systemisk lupus erytematosus (SLE) är en autoimmun sjukdom där immunförsvaret attackerar kroppsegen vävnad. På grund av varierande symptom och sjukdomsmanifestationer kan det vara svårt att skilja pågående sjukdomsaktivitet/skov från permanent organskada orsakad av tidigare skov eller biverkningar av medicinering. Det finns ett stort kliniskt behov av att identifiera nya molekyllära markörer som speglar sjukdomsaktivitet och organskada.

Sjukdomen kännetecknas av att det bildas autoantikroppar mot strukturer i cellkärnan. När dessa antikroppar binder sina målstrukturer kan det bidra till uppkomst av sjukdom. I Arbete I fann vi, i blodprover från ca 1/4 av SLE-patienterna, förekomst av autoantikroppar som binder till proteinet HMGB1, dvs. ett protein som finns i cellkärnan och som kan utsöndras till cirkulationen. Förekomst av dessa antikroppar korrelerade med sjukdomsaktivitet.

Patienter med SLE har försämrad förmåga att eliminera döda celler, vilket bidrar till att cellskräp blir kvar både i cirkulationen och i olika organ där det orsakar inflammation. Pentraxiner är en familj av proteiner som bildas i stora mängder vid inflammation och som kan hjälpa till med att städa bort döda celler. I Arbete II analyserades förekomst av cirkulerande pentraxin 3 (PTX3) hos SLE-patienter. Vi fann att detta protein hämmas av interferon (IFN)- α , en av immunförsvarets signalmolekyler vars koncentration i blodet är förhöjd hos SLE-patienter. IFN- α -medierad minskning av cirkulerande PTX3 leder sannolikt till ökade problem med cellskräpshantering, och därmed troligen till accelererad inflammation.

Osteopontin (OPN) är ett protein som är involverat i immunförsvaret där det kan påverka både cellsignalering och reglering av celldöd. OPN har föreslagits utgöra en organskademarkör hos barn med SLE. I Arbete III och IV undersökte vi om OPN är en markör för organskada och/eller sjukdomsaktivitet hos vuxna. I Arbete III fann vi att nivåerna av OPN är förhöjda hos SLE-patienter. Hos nyinsjuknade patienter speglade nivåerna av OPN graden av sjukdomsaktivitet. Hos patienter med en mer etablerad sjukdom, däremot, flaggade nivåerna av OPN istället för organskada. I Arbete IV mättes OPN i ett internationellt material med prover från 345 nyinsjuknade SLE-patienter och med fem års uppföljningsdata. I denna

studie var inte kopplingen mellan OPN och organskada tydlig, men däremot återspeglade OPN sjukdomsaktiviteten både vid sjukdomsdebut och över tid.

Sammanfattningsvis kan förmodligen ökade nivåer av autoantikroppar såsom anti-HMGB1 och minskade nivåer av PTX3 bidra till ökade problem med skräphanteringen och inflammation. Av de molekyler vi analyserat verkar OPN vara den som bäst speglar sjukdomsaktivitet. Fortsatta studier av dessa markörer kan hjälpa oss att bättre förstå sjukdomsmekanismerna vid SLE och bidra till förfinad diagnostik, och bättre indelning och uppföljning av patienter med SLE.

LIST OF PAPERS

This thesis is based on the following papers, which are referred to in the text by their Roman numerals.

Paper I

Lina Wirestam*, Hanna Schierbeck*, Thomas Skogh, Iva Gunnarsson, Lars Ottosson, Helena Erlandsson-Harris, Jonas Wetterö, Christopher Sjöwall. Antibodies against High Mobility Group Box protein-1 (HMGB1) versus other anti-nuclear antibody fine-specificities and disease activity in systemic lupus erythematosus. *Arthritis Research & Therapy*, 2015, 17:338.

*Shared first authorship

Paper II

Lina Wirestam, Helena Enocsson, Thomas Skogh, Maija-Leena Eloranta, Lars Rönnblom, Christopher Sjöwall, Jonas Wetterö. Interferon- α coincides with suppressed levels of pentraxin-3 (PTX3) in systemic lupus erythematosus and regulates leucocyte PTX3 *in vitro*. *Clinical & Experimental Immunology*, 2017, 189:83-91.

Paper III

Lina Wirestam, Martina Frodlund, Helena Enocsson, Thomas Skogh, Jonas Wetterö, Christopher Sjöwall. Osteopontin is associated with disease severity and antiphospholipid syndrome in well characterised Swedish cases of SLE. *Lupus Science & Medicine*, 2017, 4:1,000225.

Paper IV

Lina Wirestam, Helena Enocsson, Thomas Skogh, Leonid Padyukov, Andreas Jönsen, Murray Urowitz, Dafna Gladman, Juanita Romero-Diaz, Sand-Cheol Bae, Paul R Fortin, Jorge Sanchez-Guerrero, Ann Clarke, Sasha Bernatsky, Caroline Gordon, John G Hanly, Daniel Wallace, David Isenberg, Anisur Rahman, Joan Merrill, Ellen Ginzler, Graciela S Alarcón, Barri J Fessler, Michelle Petri, Munther Khamashta, Cynthia Aranow, Mary Anne Dooley, Susan Manzi, Rosalind Ramsey-Goldman, Ola Nived, Elisabet Svenungsson, Kristjan Steinsson, Asad Zoma, Guillermo Ruiz-Irastorza, Sam Lim, Ken Kalunian, Murat Inanc, Ronald van Vollenhoven, Manuel Ramos-Casals, Diane Kamen, Søren Jacobsen, Christine Peschken, Anca Askanase, Thomas Stoll, Ian N Bruce, Jonas Wetterö, Christopher Sjöwall. Osteopontin is a marker of disease activity, but not a distinct predictor of future damage in recent-onset systemic lupus erythematosus: Results from the Systemic Lupus International Collaborating Clinics inception cohort. Manuscript.

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ABBREVIATIONS

ACR	American College of Rheumatology
ANA	anti-nuclear antibody
APS	antiphospholipid syndrome
ATP	adenosine triphosphate
AU	arbitrary units
AUC	area under curve
BILAG	the British Isles Lupus Assessment Group
CRP	C-reactive protein
DELFA	dissociation-enhanced lanthanide fluorescence immunoassay
DMARD	disease-modifying anti-rheumatic drug
DNA	deoxyribonuclease
dsDNA	double-stranded deoxyribonuclease
dsRNA	double-stranded ribonucleic acid
ELISA	enzyme-linked immunosorbent assay
ESR	erythrocyte sedimentation rate
FITC	fluorescein-isothiocyanate
HLA	human leukocyte antigen
HMGB1	high mobility group box protein-1
ICs	immune complexes
IF-ANA	immunofluorescence microscopy ANA
IFN	interferon
IFNAR	IFN- α/β receptor
Ig	immunoglobulin
IL	interleukin
IRF	interferon regulatory factor
ISGF	interferon-stimulated gene factor
KLURING	Kliniskt LUPusRegister I Nordöstra Götaland (Swedish); Clinical Lupus Register in Northeastern Gothia (English)

LPS	lipopolysaccharide
MBL	mannose-binding lectin
OPN	osteopontin
PBMC	peripheral blood mononuclear cells
pDC	plasmacytoid dendritic cell
PGA	Physician Global Assessment
PTX3	pentraxin 3
RAGE	receptor of advanced glycation end products
SAP	serum amyloid P component
SDI	SLICC/ACR damage index
SELENA	the Safety of Estrogens in Lupus Erythematosus National Assessment
SLAM	Systemic Lupus Activity Measure
SLE	systemic lupus erythematosus
SLEDAI-2K	SLE disease activity index 2000
SLICC	Systemic Lupus International Collaborating Clinics
SNPs	single nucleotide polymorphisms
SRI	SLE responder index
STAT	signal transducer and activator of transcription
TBK1	TNF receptor associated factor family member-associated NF- κ B activator-binding kinase 1
TGF- β	transforming growth factor β
Th	T helper
TLR	toll-like receptor
TNF	tumor necrosis factor
TRAP	tartrate-resistant acid phosphatase
VAS	visual analog scale

INTRODUCTION

Systemic lupus erythematosus (SLE) was first clearly described in the Middle Ages when cutaneous lupus was reported by the Italian physician Rogerius Salernitanus [1]. He was the first to use lupus (Latin for wolf) erythematosus (Latin for red) to describe the facial rash which he thought resembled a wolf bite. It was first in the beginning of the 20th century that the implication of the immune system was acknowledged, when Hauck reported false positive Wasserman test (an antibody test for syphilis) among patients with SLE [2]. Since then, research regarding involvement of the immune system in SLE has evolved tremendously.

SLE is an autoimmune disease, i.e. the immune system fails to discriminate 'self' from 'non-self' eventually leading to attack on 'self tissues' [3]. Autoimmune diseases are thought to result from a combination of genetic susceptibility, infection and environmental triggers and breakdown of checkpoints in self-tolerance.

SLE CLASSIFICATION

SLE is a complex heterogeneous disease where most organ systems can be affected. The disease course often shows periods of relapses followed by remission. General vague symptoms, like fatigue, are common. Otherwise, the most frequent manifestations are skin and joint involvement, which are present in a majority (>80%) of SLE patients, at least at some occasion during the disease course [4].

The 1982 classification criteria for SLE presented by the American College of Rheumatology (ACR) [5] are listed in Table 1. To be classified as SLE ≥ 4 of the ACR-82 criteria must be fulfilled. The ACR-82 criteria were revised by the Systemic Lupus International Collaborating Clinics (SLICC) group in 2012 in order to improve clinical relevance and incorporate new immunological items [6]. The SLICC-12 criteria (Table 2) require (i) either fulfilment of ≥ 4 criteria of which at least 1 clinical and 1 immunologic criterion must be present, or (ii) biopsy-

proven nephritis in combination with positive test for antinuclear antibodies (ANA) and/or anti-double-stranded deoxyribonuclease (dsDNA) antibodies.

Table 1. 1982 classification criteria for systemic lupus erythematosus.

Requirements: ≥4 criteria presented serially or simultaneously	
Criterion	Definition
1. Malar rash	Erythema over the malar eminences
2. Discoid rash	Erythematous raised patches
3. Photosensitivity	Skin rash as a result of unusual reaction to sunlight
4. Oral ulcers	Oral or nasopharyngeal ulceration
5. Arthritis	Non-erosive arthritis involving 2 or more peripheral joints
6. Serositis	Pleuritis or pericarditis
7. Renal disorder	Proteinuria or cellular casts
8. Neurologic disorder	Seizures or psychosis
9. Hematologic disorder	Hemolytic anemia or leukopenia or lymphopenia or thrombocytopenia
10. Immunologic disorder	Positive LE cell preparation or abnormal serum level of anti-dsDNA or anti-Sm or Wasserman reaction
11. Antinuclear antibody	ANA in abnormal level and absence of drugs associated with ‘drug-induced lupus’ syndrome

ANA = antinuclear antibodies, dsDNA = double stranded deoxyribonucleic acid, LE = Lupus erythematosus, Sm = Smith.

Summarized 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(11):1271-7. [5]

Table 2. SLICC classification criteria for systemic lupus erythematosus.

Requirements: ≥4 criteria, at least 1 clinical and 1 immunologic criteria OR biopsy-proven nephritis with positive ANA or anti-dsDNA	
Clinical criteria	Immunological criteria
1. Acute cutaneous lupus	1. ANA *
1. Chronic cutaneous lupus	2. Anti-dsDNA *
2. Oral or nasal ulcers	3. Anti-Sm *
3. Non-scarring alopecia	4. Anti-phospholipid antibodies *
4. Arthritis	5. Low complement
5. Serositis	6. Direct Coombs’ test in the absence of hemolytic anemia
6. Renal	
7. Neurological	
8. Hemolytic anemia	
9. Leukopenia or lymphopenia	
10. Thrombocytopenia	

ANA = antinuclear antibodies, dsDNA = double stranded deoxyribonucleic acid, Sm = Smith, * at abnormal serum levels.

Summarized from Petri M, et al. Derivation and validation of the Systemic Lupus International Collaborating Clinics classification criteria for systemic lupus erythematosus. *Arthritis Rheum.* 2012;64:2677-86. [6]

NATURE AND NURTURE

The prevalence of SLE varies between ethnic groups. In Sweden the prevalence rate is about 65-80 cases per 100,000 inhabitants [7, 8]. The incidence is highest among women during childbearing years, with a female:male ratio of 9:1 [7, 8]. It is unknown why females are more susceptible to SLE, but estrogen is likely to contribute to systemic inflammation in SLE [9, 10]. Furthermore, the cause may depend on the X-chromosome. Normally one of the X-chromosomes is silenced in females, but this inactivation is not complete, and about 15% of the genes escape the silencing process. Many immune-related genes like CD40 ligand, toll-like receptor (TLR) -7 and -8 are located on the X-chromosome, and an overexpression of these gene products may influence the immune response [10].

The cause/causes of SLE is/are unknown, but both genetic and environmental factors contribute to development of the disease. Studies among twins have shown a ten-fold higher risk to develop SLE for monozygotic twins compared to dizygotic [11]. Genome-wide association studies have revealed >60 risk loci for SLE susceptibility [12]. Strong associations are found within the human leukocyte antigen (HLA) region, especially with HLA-DRB1 [11]. Other non-HLA genes involved in SLE susceptibility are associated with interferon (IFN) signaling (e.g. IFN regulatory factor (IRF) 5, 7, 8, and signal transducer and activator of transcription (STAT) 4), complement deficiencies (later mentioned), and antigen receptor signal transduction in B- and T-cells (i.e. PTPN22).

Environmental factors like UV-light and smoking are associated with a higher risk, while a small consumption of alcohol may protect against SLE development [13]. Epstein-Barr virus infection has also been proposed as a disease trigger [14].

DISEASE ACTIVITY AND ORGAN DAMAGE

Disease activity can be defined as reversible manifestations of underlying inflammatory processes. The diversity of disease phenotypes among patients with SLE makes it hard to find reliable biomarkers that adequately reflect disease activity and/or organ damage.

There are several assessment systems available for disease activity, e.g. the British Isles Lupus Assessment Group (BILAG) [15, 16], Systemic Lupus Activity Measure (SLAM) [17], Physician Global Assessment (PGA), SLE Disease Activity Index (SLEDAI) [18], the Safety of Estrogens in Lupus Erythematosus National Assessment (SELENA)-SLEDAI and SLE responder index (SRI) [19]. SLAM, PGA and SLEDAI are global indices providing an overall measure of activity. The SELENA-SLEDAI was elaborated for clinical trials with the intention to distinguish severe from mild flares. BILAG, on the other hand, evaluates disease activity in 8 separate organ systems. PGA is a visual analog scale (VAS) ranging from 0 to 3. The SRI combines the SELENA-SLEDAI to cover global disease, BILAG to cover organ specific disease and PGA as a validity and for items that were not covered by the other two indexes [19].

Throughout the thesis, the SLEDAI 2000 (SLEDAI-2K) was used to assess disease activity (Table 3). This index is composed of a combination of clinical and laboratory data, where the physician decides whether each manifestation has been present or absent during the last 10 days [20]. SLEDAI-2K is a global weighted tool, i.e. the descriptors are multiplied by their 'weight'. For example, arthritis is multiplied by four, and central nervous descriptors are multiplied by eight. The weighted descriptors are then summed into the final score, which can range from zero to 105. Based on SLEDAI scoring, activity categories have been defined; A score of zero means 'no activity', while a score of 1-5 represents 'mild activity' [21]. 'Moderate activity' is reflected by a score between 6-10 and a score above 11 reflects 'high activity'. Moreover, an increase in SLEDAI-2K score of more than 4 is usually categorized as a 'flare'.

Table 3. SLEDAI-2K descriptors and scores.

SLEDAI-2K score	Descriptor	Definition
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 with impaired orientation, memory or other intellectual function.
8	Visual disturbance	Retinal changes.
8	Cranial nerve disorder	New onset of sensory or motor neuropathy involving cranial nerves.
8	Lupus headache	Severe, persistent headache which 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 of vasculitis.
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 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.
2	Increased DNA binding	Increased DNA binding by Farr assay.
1	Fever	>38°C. Exclude infectious cause.
1	Thrombocytopenia	<100 000 platelets / $\times 10^9/L$, exclude drug causes.
1	Leukopenia	<3000 white blood cells / $\times 10^9/L$, exclude drug causes.

C3 = Complement protein 3, C4 = Complement protein 4, CH50 = 50% hemolytic complement activity, DNA = deoxyribonuclease, SLEDAI-2K = SLE disease activity index 2000

Summarized from Gladman DD, Ibanez D, Urowitz MB. *Systemic lupus erythematosus disease activity index 2000. The Journal of Rheumatology.* 2002;29:288-91. [18]

The SLICC/ACR damage index (SDI) is a tool for assessing accumulated organ damage in SLE [22] (listed in Table 4). It includes 12 organ systems and records damage that may result from previous disease activity leading to organ failure, or from medication side-effects. To distinguish damage from active inflammation, it must be a non-reversible change present for at least 6 months. The SDI of a patient can range from 0 to a maximum score of 45. Once a SDI item is recorded it is permanent, and consequently the score cannot decrease. It is known that the SDI value is a good predictive value of both survival and mortality [23].

Table 4. SLICC/ACR Damage Index (SDI).

Non-reversible change present for at least 6 months		
Organ system	Example of damage	Maximum score
Ocular	Cataract	2
Neuropsychiatric	Cerebrovascular accident	6
Renal	Glomerular filtration rate <50%	3
Pulmonary	Pulmonary hypertension	5
Cardiovascular	Myocardial infarction	6
Peripheral vascular	Venous thrombosis	5
Gastrointestinal	Infarction or resection of bowel below duodenum, spleen, liver or gall bladder	6
Musculoskeletal	Osteoporosis with fracture	6
Skin	Scarring chronic alopecia	2
Premature gonadal failure		1
Diabetes		1
Malignancy		2

Summarized from Gladman D, Ginzler E, Goldsmith C, Fortin P, Liang M, Urowitz M, et al. The development and initial validation of the Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index for systemic lupus erythematosus. *Arthritis Rheum.* 1996;39:363-9. [22]

THERAPY

Most SLE patients are prescribed the ‘antimalarial drug’ hydroxychloroquine as a basic treatment. Hydroxychloroquine is associated with improved survival and is effective, especially against arthritis and cutaneous disease [24]. Other disease-modifying anti-rheumatic drugs (DMARDs) used in SLE are azathioprine, methotrexate, cyclophosphamide and cyclosporine, depending on which type(s) of manifestations the clinician aim to treat [25]. Combination of DMARDs with corticosteroids are often prescribed. Due to side effects, the

aim is to taper off or at least minimize the dose of corticosteroids [26]. In SLE, B-cells play a pivotal role in presenting autoantigens to T-cells leading to cytokine release and autoantibody formation. This has led to the search for B-cell targeting therapies. At present, rituximab (anti-CD20) and belimumab (anti-B lymphocyte stimulating factor; anti-BLyS) are the two most commonly used biologic DMARDs in SLE [24, 27]. Since the type I Interferon system is crucial in the SLE pathogenesis, therapies interfering with IFN- α furthermore appears to be a logical approach. Anifrolumab, an antibody that binds to the type I IFN receptor thereby blocking IFN- α signaling, seems promising [28].

PATHOGENESIS AND THE WASTE-DISPOSAL THEORY

The pathogenesis of SLE is characterized by dysregulation of the immune system. When cells undergo apoptosis, it is extremely important that debris is rapidly removed. Apoptotic cells that are not sufficiently eliminated may undergo secondary necrosis, thereby releasing autoantigens and endogenous danger signals such as high mobility group box protein-1 (HMGB1), uric acid and adenosine triphosphate (ATP), that promote inflammation. Compared to healthy controls, patients with SLE display impaired phagocytosis by granulocytes and monocytes [29]. Defective clearance of cellular debris may be responsible for the initiation of SLE, leading to increased antigen exposure, which in turn promotes autoantibody production. Furthermore, autoantibodies and antigens form immune complexes (ICs) which can induce production of IFN- α by plasmacytoid dendritic cells (pDC). Deficient physiologic clearance of circulating ICs can lead to IC-deposition in tissues, e.g. kidneys, and cause inflammation *in situ*. In this way, a vicious circle of increased apoptotic rate, impaired clearance, autoantigen exposure, autoantibody production, chronic inflammation and tissue damage goes on.

The elimination of apoptotic cells by phagocytosis is enhanced by opsonizing proteins such as pentraxins, C1q, immunoglobulin (Ig) G and mannose-binding lectin (MBL). Genetic deficiency of early complement proteins, like C1q and C4, leads to a very high risk of developing SLE [30]. Although such deficiencies are rare, many patients acquire a deficiency due to consumption of proteins as the disease progresses.

A hallmark of SLE is the occurrence of autoantibodies targeting nuclear antigens, i.e. ANA. ANA is a large family of autoantibodies which can be further subdivided based on distinct immuno-morphological immunofluorescence microscopy ANA (IF-ANA) staining patterns, e.g. *homogenous pattern* due to reactivity with chromatin-associated antigens (e.g. double-stranded DNA, histones or DNA-histone complexes), *speckled pattern* reflecting antibodies to extra-chromosomal antigens (e.g. various ribonucleoproteins), *nucleolar pattern* due to reactivity with nucleolar proteins, and *centromeric pattern* corresponding to antibody affinity for centromeric proteins [31]. The most common IF-ANA staining pattern in SLE is homogenous, followed by speckled ANA [32].

IFN- α

The type I interferon system is an important part of our defense against viral infections and consists mainly of IFN- α and IFN- β [33]. IFN production is triggered by viruses and double-stranded ribonucleic acid (dsRNA), which are sensed by TLRs (Figure 1A). TLR-3 recognizes dsRNA, whereas TLR-7 and -8 are activated by single-stranded ribonucleic acid (ssRNA) [34]. Furthermore, unmethylated CpG-rich DNA trigger TLR-9 activation. Most TLRs signal through the MyD88 adaptor proteins which recruit the kinases IKK ϵ and tumor necrosis factor (TNF) receptor associated factor family member-associated NF- κ B activator -binding kinase 1 (TBK1). The kinases then phosphorylate transcriptional factors, including IRF3, IRF5 and IRF7. The IRFs, together with additional transcriptional factors, initiate expression of the IFN- and IRF-induced genes with subsequent production of IFN- α . The IFN- α/β receptor (IFNAR), which is expressed by almost all cell types, consists of two subunits; IFNAR-1 (α -subunit) and IFNAR-2 (β -subunit) [35]. Binding of IFN- α activates Jak-1 and Tyk-2, which phosphorylates STAT proteins (Figure 1B). Classically STAT1 and STAT2 form a homodimer which associates with IRF9, resulting in the (interferon-stimulated gene factor 3) ISGF3 complex. Further on, ISGF3 translocates to the nucleus and activates interferon response elements [35].

The main IFN- α producing cells are the pDCs [36], which respond to viral nucleic acids via endosomal TLR-7 and -9 by massive IFN- α production. Although most cell types can produce IFN- α , pDCs can produce 10-100 times more (up to 10^9 IFN- α molecules per cell in 24h). The

majority of patients with SLE have raised circulating levels of IFN- α , and/or express IFN-inducible genes, i.e. ‘the type I IFN signature’ [37, 38]. Normally pDCs are activated by bacterial or viral nucleic acids. In SLE, however, they are activated by DNA- and RNA-containing ICs via Fc γ -receptor-dependent uptake, followed by endosomal TLR-7 and -9 activation [36]. The importance of IFN- α in SLE is further displayed by the fact that administration of IFN- α in viral infections or cancer can induce a lupus-like disease [39].

IFN- α has many effects on the immune system (reviewed in [37]) such as enhanced antigen presentation by dendritic cells and suppression of regulatory T-cells. Furthermore, the effects on B-cells include increased plasma cell differentiation, isotype switch and boosted production of antibodies.

Figure 1. A) IFN- α production in SLE. Immune complexes (ICs) containing nucleic acids are internalized to endosomes via Fc gamma receptor IIa (Fc γ IIa) where they bind to toll like receptors (TLRs). Activation of TLR-7 and/or -9 will lead to the expression of NF κ B and IRF7, with IFN- α and other pro-inflammatory end products. **B)** Binding of IFN- α to its receptor initiates activation of different members of the signal transducer and activator of transcription (STAT)-family. STAT1, STAT2 and IRF9 constitutes the interferon-stimulated gene factor 3 (ISGF3) complex, which binds to IFN-stimulated response elements (ISRE), resulting in antiviral responses. STAT1 homodimers binds to gamma-activated sequences (GASs) inducing pro-inflammatory responses. STAT3 homodimers on the other hand, leads to anti-inflammatory responses via GASs.

HMGB1

HMGB1 is a nuclear protein capable of binding to DNA, thereby regulating gene transcription, chromatin replication and DNA repair [40]. HMGB1 consists of three domains; A box, B box, and an acidic tail. It is expressed by almost all cells and can be actively released by immune cells (e.g. monocytes and dendritic cells) [41, 42]. Normally, during apoptosis, HMGB1 is not released, but during secondary necrosis HMGB1 attached to DNA will be released to the extracellular milieu [43]. HMGB1 is cytokine-inducing, but not when bound to DNA due to irreversible oxidation of the critical cysteine 106 that is essential for HMGB1 to stimulate cytokine production [44]. Yet, HMGB1 attached to DNA, or by itself, is highly immunogenic as a nuclear autoantigen irrespective of its redox state. HMGB1 can bind to multiple receptors, including the receptor for advanced glycation end products (RAGE), TLR-2, -4 and -9. ICs containing HMGB1 can activate the TLR-2 pathway to induce production of anti-dsDNA antibodies [45]. Furthermore, HMGB1 bound to DNA-containing ICs has also been shown to enhance IFN- α production by pDCs, and to activate autoreactive B cells [46]. HMGB1 can also skew the differentiation of M2-like macrophages to the more pro-inflammatory M1-like phenotype, and decrease phagocytosis of apoptotic cells [47]. In SLE, serum levels of HMGB1 have been shown to be elevated and correlated with disease activity [40, 48-50].

PTX3

The pentraxin protein family consists of two subgroups; short pentraxins (i.e. C-reactive protein (CRP) and serum amyloid P component (SAP)) and long pentraxins (e.g. pentraxin 3 (PTX3)) [51]. PTX3 is structurally and functionally related to CRP but its production differs both with regard to its non-hepatic cell origin as well as to inducing stimuli. The 440 kDa protein is composed of eight identical protomers associated through disulfide bonds [52]. Its production by monocytes and macrophages is induced by lipopolysaccharide (LPS) and interleukin (IL) - 1 β [53, 54], and the release of stored PTX3 from neutrophils is triggered by LPS and TNF [53, 55].

A number of single nucleotide polymorphisms (SNPs) in the PTX3 gene have been found [51]. Some SNPs have been reported to associate with altered blood levels of PTX3 when

comparing patients with acute myocardial infarction with controls [56]. Variants of the SNP *rs2305619* have been associated with differences in PTX3 plasma levels both at baseline, and 24h after lung transplantation [57].

PTX3 contributes to the waste disposal of cell debris as well as the elimination of pathogens via C1q and classical complement activation [51]. Furthermore, binding of PTX3 to apoptotic cells can inhibit recognition by dendritic cells, thereby maintaining peripheral immune tolerance. Another feature of PTX3 is the ability to induce macrophage secretion of immunosuppressive cytokines, such as IL-10 and transforming growth factor (TGF) - β .

Because of failed waste-disposal handling in SLE, it is not farfetched to consider PTX3 as a significant player in this vicious circle. Low levels of pentraxins, like CRP and PTX3 could thus result in accumulation of cell debris and subsequent inflammation and autoimmunity [58, 59]. Previous reports have reported both elevated [60-62] and lowered [63-65] levels of PTX3 in SLE patients, as compared to control subjects.

CRP-supplementation to lupus model mice leads to decreased levels of autoantibodies, less autoimmune manifestations and enhanced survival [66]. On the other hand, a recent study demonstrated that immunization with PTX3 in a murine lupus model lead to anti-PTX3 antibody production, which delayed lupus-like nephritis and prolonged the survival [67].

OPN

Osteopontin (OPN) is an extracellular matrix protein with multiple functions. It was first identified in bone mineralization and remodeling. Moreover, it has been suggested to be important in other biological processes like vascular calcification and immune cell signaling [68]. It is produced by various cells including B- and T- cells, dendritic cells, macrophages, neutrophils, bone cells and neurons, in response to injury and inflammation [69]. OPN has been shown to inhibit apoptosis of inflammatory cells, for example in the synovial fluid from RA patients OPN 'rescues' activated T cells [70]. Moreover, OPN interacts with a variety of cell surface receptors, including the receptor (CD44) for hyaluronic acid, and binding to these receptors stimulates cell adhesion, activation and migration [71].

Increased levels of OPN have been found in SLE patients compared to controls [72], and to correlate with both disease activity [73] and damage accrual [74, 75]. Mechanistic implications for a role in SLE pathogenesis includes that overexpression of OPN in lupus prone mice induces B-cell activation and subsequent production of anti-dsDNA antibodies [76, 77]. Furthermore, intracellular expression of OPN in pDC is required for TLR-9-dependent expression of IFN- α [78].

The general aim of this thesis project was to investigate and evaluate potential new biomarkers of disease activity and/or organ damage in SLE.

The aims of the specific projects are listed below:

- To detect serum anti-HMGB1 antibodies and relate them to other types of antinuclear antibodies (ANA), and to disease activity (Paper I).
- To investigate PTX3 levels in patients, and to determine whether PTX3 is influenced by IFN- α both *in vitro* and *in vivo* (Paper II).
- To investigate OPN as a potential marker of organ damage and/or disease activity (Paper III and IV).

A graphical overview of the thesis project is shown in Figure 2.

Figure 2. Overview of the thesis project with hypothetical interactions. Patients with SLE display an increased apoptosis rate in combination with an impaired clearance. Proteins might leak out from the apoptotic cells, like HMGB1, and autoantibodies may be formed (Paper I). SLE patients have increased levels of IFN- α , which could interfere with PTX3 (Paper II). PTX3 contributes to the waste disposal and low levels could thus result in accumulation of cell debris and subsequent inflammation in a vicious circle. SLE patients have elevated levels of OPN (Paper III & IV). Studies in mice have shown that OPN can stimulate IFN- α production and also inhibit apoptosis, which could fuel the waste-disposal problem, with organ damage as future outcome. Green arrow indicates stimulation, red inhibition, and black unknown effect.

COMMENTS ON MATERIALS & METHODS

KLURING

Throughout the thesis, I have had the opportunity to work with our cohort designated KLURING, which is a Swedish acronym for 'Kliniskt LUpusRegister I Nordöstra Götaland'. KLURING was initiated in 2008 to start up an SLE register and a biobank in this area of Sweden. Each patient >18 years of age who had an SLE diagnosis based on either at least 4 of the ACR-82 criteria or cases who had a positive ANA test in combination with typical symptoms from at least two organ systems (the 'Fries' diagnostic principle'), are since then asked to contribute to the cohort. Thus, both incident and prevalent cases were asked to enroll.

At inclusion, an extra blood sample for genetic analyses was stored. At each visit, according to normal clinical routine, extra serum (and more recently also plasma) samples are saved in the biobank for future research. Clinical routine analyses (e.g. CRP, erythrocyte sedimentation rate (ESR), leukocyte variables, anti-dsDNA, complement components/activity and urinalysis) and registration of disease activity, organ damage and medications are registered in a database at every revisit. Also, patient-reported outcome measures including longitudinal data on quality of life, pain and fatigue are included.

In 2017, the cohort contained about 280 patients.

Serum was prepared and stored at -70°C and thereafter thawed and divided into aliquots. OPN, anti-HMGB1 and PTX3 were analyzed from aliquots that had been freeze-thawed 2-3 times.

Patients in Paper I, II and III were selected based on fulfilment of the ACR-82 and/or SLICC-12 criteria.

SLICC INCEPTION COHORT

The SLICC group was formed in 1991 by rheumatologists interested in assessing disease activity and organ damage. The SLICC group comprises 31 centers from 11 countries in North America, Europe and Asia. In year 2000, the SLICC group started an inception cohort which has over 1600 newly diagnosed patients enrolled within 15 months of diagnosis and with annual follow-up of up to 10 years for some patients [79]. In Paper IV, I had the opportunity to analyze sera from 345 patients included in the SLICC inception cohort with recent-onset disease, all fulfilling the ACR-82 criteria.

ANTI-HMGB1 ANTIBODY ELISA

The anti-HMGB1 enzyme-linked immunosorbent assay (ELISA) used in Paper I is an in-house ELISA, where plates are coated with rat histidine-tagged HMGB1. The production of recombinant HMGB1 was performed at the Karolinska Institute, Stockholm. HMGB1 is a highly conserved protein with 99% identity between mammals [80]. The differences between human and rat HMGB1 reside in the highly acidic C-terminal tail, in which the amino acids in position 189, 202 and 215 are substituted from aspartic acid to glutamic acid, or vice versa. The rat protein is fully functional in human systems with no differences in reactivity reported in the literature.

INDIRECT IMMUNOFLUORESCENCE MICROSCOPY FOR ANA PATTERNS

Abnormally high serum levels of ANA assessed by indirect immunofluorescence microscopy (IF-ANA) is one of the 11 classification criteria for SLE according to the ACR-82 [5], and still recommended as the basic method for ANA detection (SLICC-12). In Paper I, IF-ANA was used for visualization of ANA. Applying the stipulated cut-off level, i.e. >95th percentile among healthy female blood donors, IF-ANA occurs in the large majority (98-99%) of SLE patients at diagnosis [81]. Depending on the nuclear target for the different ANA specificities, different

nuclear IF-staining patterns can be envisioned [82]. Antibodies against dsDNA, histones and DNA-histone complexes typically produce a homogenous nuclear staining pattern in non-dividing cells, and staining of the condensed chromatin-associated antigens in mitotic cells (Figure 3). In contrast, ANA specific for extrachromosomal antigens can be recognized as a speckled nuclear staining pattern in non-dividing cells, and diffuse extra-chromosomal staining of dividing cells. Other staining patterns (e.g. centromeric, nucleolar, nuclear dots and nuclear membrane) can be distinguished and indicate other antigen-specificities. In SLE, the homogenous IF-ANA pattern predominates, followed by speckled IF-pattern.

Figure 3. Principles and patterns of immunofluorescence anti-nuclear antibodies (IF-ANA) used in Paper I. Fixed/permeabilized HEP-2 cells are incubated with patient serum. If the serum contains ANA, they will bind to nuclear antigens. A secondary antibody conjugated with a fluorescent marker will then visualize the ANAs as different immuno-morphological staining patterns (e.g. homogenous, centromeric, speckled, nucleolar, nuclear dots and nuclear membrane) depending on the ANA specificities.

ANA FINE-SPECIFICITY

After IF-ANA is performed, analyses of ANA fine-specificities (i.e. analyses of specific autoantibodies) are often carried out. This can be done by Western Blot, ELISA, immunoprecipitation or other techniques. In Paper I we analyzed ANA fine-specificities by a line blot technique (immunoblot) from EUROIMMUN (ANA Profile 5, EUROIMMUN, Lübeck, Germany). The technique uses strips coated with parallel lines of 18 different antigens (Figure 4). Detection antibodies are conjugated with an enzyme converting its substrate into a colored product. Stripes are scanned with EUROLinScan and you obtain both semi-quantitative and qualitative data based on densitometry. However, the manufacturer itself refers to it as a qualitative test.

Figure 4. Immunoblot strip for analysis of fine-specificities. The strips are coated with parallel lines of 18 antigens. In paper I, this method is used to evaluate dsDNA, nucleosomes and histones.

PTX3 ELISA

The PTX3 ELISA used in Paper II, from R&D Systems (Minneapolis, MN, USA) is verified for plasma. Since only serum samples were available from KLURING, we performed a correlation study. Serum and plasma samples were collected simultaneously from 6 SLE cases. The results of plasma and serum samples showed very high correlation ($r=0.97$, $p=0.001$) (Figure 5).

Figure 5. Pearson correlation of plasma and serum pentraxin 3 (PTX3) collected simultaneously in 6 SLE patients and measured by enzyme-linked immunosorbent assay (ELISA). $r=0.97$ $p=0.001$ r = Pearson correlation coefficient.

OPN ELISA

The OPN ELISA used in Paper III and IV (from R&D Systems) is validated for plasma and serum. Since only serum samples were available from KLURING and the SLICC inception cohort, we performed a correlation study. Serum and plasma samples were collected simultaneously from 8 individuals (6 women, 2 men; mean age 34.5 years; range 24-46 years). Plasma and serum samples correlated ($r=0.77$, $p=0.027$), however, the levels of OPN tended to be somewhat lower in the serum samples (Figure 6). This is in line with the validation data by the manufacturer.

Figure 6. Pearson correlation of plasma and serum osteopontin (OPN) collected simultaneously in 8 individuals and measured by enzyme-linked immunosorbent assay (ELISA). $r=0.77$ $p=0.027$ r = Pearson correlation coefficient.

DELFI

In Paper II we used a dissociation-enhanced lanthanide fluorescence immunoassay (DELFI) to detect IFN- α . The assay was performed at Uppsala University. DELFI uses lanthanide chelates (Europium), which has a longer fluorescence decay time compared to traditional fluorophores. Time-resolved fluorescence measurement is used instead of normal fluorescence intensity measurement, rendering a lower autofluorescence. DELFI offers a high sensitivity and a wide dynamic range.

CELL VIABILITY

A tetrazolium-based assay was used to determine the relative number of viable cells (CellTiter 96 Aqueous One Solution Cell Proliferation Assay, Promega, Madison, WI, US) in Paper II. It is a colorimetric method for determining the amount of viable cells. The tetrazolium compound is bio-reduced (probably by NADPH) by metabolic active cells into a colored formazan product, and the absorbance can be measured.

SUMMARY OF PAPERS

PAPER I

Anti-HMGB1 antibodies occur in SLE and correlate with some disease activity variables. Although anti-HMGB1 antibodies measured by ELISA often coincide with a homogenous staining pattern, our results indicate that anti-HMGB1 antibodies do not give rise to nuclear staining of the widely used HEp2-cell IF-ANA microscopy slides.

PAPER II

PTX3 contributes to the waste disposal of cell debris. *In vitro*, IFN- α exerts diverse effects on neutrophils and peripheral blood mononuclear cells (PBMC), leading to release of PTX3 from neutrophils and attenuated synthesis of PTX3 from PBMC. The net effect is probably reduced levels since SLE patients with raised circulating IFN- α have reduced levels of circulating PTX3. Given the important role of PTX3 in the clearance of dying cells and the prominent activation of the type I IFN system in SLE, our results suggest that the suppressed PTX3 levels in SLE contribute to the autoimmune disease process by providing autoantigens to B-cells and endogenous IFN inducers to pDC, which all further sustain the disease.

Increased levels of OPN have been found in SLE patients, and proposed as a marker of both disease activity and damage accrual. Our cross-sectional analyses imply OPN as a marker of disease activity at recent-onset disease. In primarily established cases of SLE, OPN appears to reflect damage accrual and cardiovascular damage. The association with antiphospholipid syndrome (APS) may predominantly be related to the damage occurring in connection with arterial events.

PAPER IV

Increased levels of OPN have been suggested to predict organ damage. Associations were found with disease activity, at inclusion as well as over time. We could not confirm previous findings by others, suggesting that OPN precede organ damage, and OPN levels could not identify new SLE cases at risk of developing future organ damage over 5 years.

RESULTS & DISCUSSION

PAPER I

Antibodies against High Mobility Group Box protein-1 (HMGB1) versus other anti-nuclear antibody fine-specificities and disease activity in systemic lupus erythematosus

The nuclear protein HMGB1 can be released extracellularly and participate in systemic inflammation [41, 42]. Administration of monoclonal anti-HMGB1 antibodies have shown opposing effects in animal models of lupus [83, 84]. In animal models of arthritis anti-HMGB1 treatment have attenuated the disease [85, 86]. The occurrence of anti-HMGB1 antibodies in SLE has previously been reported [50] and the aim of this study was to detect serum anti-HMGB1 antibodies in patients with SLE, and relate them to other types of ANAs, and to disease activity.

At inclusion 23% of the SLE patients were anti-HMGB1 positive compared to 5% of the controls. The average level of anti-HMGB1 antibodies was higher among the SLE patients (median 132.5 arbitrary units, AU) compared to the controls (median 81 AU; $p < 0.0001$).

Abnormally high levels of ANA in serum assessed by IF is one of the eleven classification criteria according to ACR-82 [5]. Only 66% of the KLURING SLE patients were IF-ANA positive, which at first may appear bothering, but is in line with findings by others [87]. The IF-ANA positivity may change over time. Most of the ANA-positive patient sera, 74%, produced a homogenous (chromosomal) IF-ANA pattern, thus 26% had other (extra-chromosomal) IF-ANA staining patterns. IF-ANA positive patients with a homogenous staining pattern had significantly higher levels of anti-HMGB1 antibodies (median 180 AU) compared to patients who were IF-ANA negative (median 83 AU) (Figure 7).

Figure 7. Indirect immunofluorescence (IF) microscopy for antinuclear antibodies (ANA) and anti-high mobility group box protein-1 (HMGB1) antibodies. Serum levels determined by ELISA of anti-HMGB1 among systemic lupus erythematosus patients grouped according to IF patterns of ANA. There were 34 % ANA negative and 66 % ANA-positive patients. IF-ANA-positive patients with a homogenous nuclear staining pattern had significantly higher levels of anti-HMGB1 antibodies compared to the IF-ANA negative group. Dashed line indicates cut-off level for a positive anti-HMGB1 test; solid lines represent median anti-HMGB1 levels. Note the axis break. (AU: arbitrary units.)

Since anti-HMGB1 positive patients were more prone to yield a homogenous pattern, it is not farfetched to assume anti-HMGB1 as a probable source of homogenous IF-ANA pattern. Hence, for immunomorphological localization of HMGB1, we incubated fixed HEp2-cells and non-fixed cryostat sections of rat liver with polyclonal rabbit anti-HMGB1. Surprisingly, we could not observe any nuclear staining on the HEp2-cells, rather a diffuse cytoplasmic staining (Figure 8). In unfixed rat liver cryostat sections a faint IF-ANA reaction with a homogenous nuclear staining pattern was observed in the hepatocytes, indicating nuclear localization of HMGB1. HEp2-cells are derived from a human adenocarcinoma, and the location of HMGB1 may predominate in the cytoplasm of malignant cells [88, 89], which could be a possible explanation for the cytoplasmic staining. Moreover, it could be a fixation artefact with a redistribution of the nuclear localized HMGB1 out to the cytoplasm. The importance of fixatives regarding distribution of cellular antigens has been highlighted previously [90-92], including HMGB1 [93]. Whatever the reasons are for the discrepancies in staining patterns between fixed HEp2-cells and unfixed rat liver sections, it is not likely that serum anti-HMGB1

antibodies will be identified as a typical positive IF-ANA pattern when using commercial HEp2-cell substrates, the predominating clinical IF-ANA test worldwide.

Figure 8. Indirect immunofluorescence microscopy targeting the cellular localization of high mobility group box protein-1 (HMGB1). **A)** Cytoplasmic/extra-chromosomal HEp-2 cell immunofluorescence after incubation with rabbit IgG anti-HMGB1 followed by fluorescein-isothiocyanate (FITC)-conjugated anti-rabbit IgG. **B)** Negative control: HEp-2 cells incubated with FITC-conjugated anti-rabbit IgG alone. **C)** Hepatocyte nuclear fluorescence after incubation of rat liver cryostat section with rabbit anti-HMGB1 and FITC anti-rabbit IgG.

We also investigated potential associations of anti-HMGB1 antibodies with different disease activity variables. Anti-HMGB1 antibodies correlated positively with anti-dsDNA antibodies ($r=0.49$, $p<0.001$), and a low correlation was found with SLEDAI-2K ($r=0.15$, $p=0.04$). The biological roles of anti-HMGB1 antibodies are far from elucidated. Studies in animal models of arthritis have shown that treatment with anti-HMGB1 antibodies can attenuate disease by blocking pro-inflammatory actions of HMGB1 [85, 86]. Opposing effects of anti-HMGB1 have been demonstrated in two different murine lupus models [83, 84]. HMGB1 has different

actions depending on if it is actively or passively released. Actively secreted HMGB1 is acetylated, and thereby cytokine-inducing, in contrast to the passively released HMGB1 [44, 94]. To study the molecular isoforms and the antibody fine-specificities could identify the source of HMGB1 in SLE.

PAPER II

Interferon- α coincides with suppressed levels of pentraxin-3 (PTX3) in systemic lupus erythematosus and regulates leucocyte PTX3 *in vitro*

The lack of correlation between CRP and disease activity in SLE [95], is partly explained by inhibition by IFN- α [96, 97]. Since PTX3 is structurally and functionally related to CRP when it comes to waste-handling actions, but derived from other cellular sources, we aimed to investigate PTX3 levels and to determine whether PTX3 is influenced by IFN- α both *in vitro* and *in vivo*.

Serum levels of PTX3 were found to be 44% lower among SLE patients compared to controls (Figure 9). Patient sera were also genotyped for three PTX3 SNPs previously reported to associate with PTX3 levels. However, no differences in PTX3 levels were observed between the SNPs. Initially, we found a low negative correlation between IFN- α and PTX3. Hence, we stratified patients into two groups with or without detectable levels of IFN- α . The PTX3 levels in patients without detectable levels of IFN- α were 29% higher compared to patients with detectable IFN- α (Figure 9). Patients without detectable levels of IFN- α also displayed lower PTX3-levels than the controls. This could be explained by the fact that many patients with increased type I IFN signature lack measurable IFN- α in serum [98, 99]. The effects of IFN- α may be performed locally or in very low concentrations that is hard to measure properly. In

Figure 9. Serum pentraxin 3 (PTX3) levels determined by enzyme-linked immunosorbent assay (ELISA) in healthy controls and patients with systemic lupus erythematosus (SLE). Serum levels of PTX3 were significantly lower in the patients with SLE (median 2.5 ng/ml) compared to the healthy controls (median 4.5 ng/ml). Patients without detectable interferon (IFN)- α (<1 U/ml) showed significantly higher levels (median 2.7 ng/ml) of PTX3 compared with patients with detectable IFN- α (>1 U/ml) (median 2.1 ng/ml). Solid lines represent median values. Note axis break.

addition to IFN- α -dependent inhibition of PTX3, consumption by e.g. autoantibodies directed towards PTX3 and tissue deposition could possibly also explain low circulating levels.

Anti-PTX3 antibodies have been reported in SLE [100]. Future studies may include analyses of anti-PTX3 antibodies to address the impact of such autoantibodies on PTX3-levels. Our group has previously investigated anti-CRP antibodies in SLE, and the typically low levels of circulating CRP in SLE-patients during flare were not explained by circulating anti-CRP antibodies [101]. Could this also be equivalent for anti-PTX3 autoantibodies? It would also be interesting to measure the type I IFN signature in the KLURING cohort and see how it relates to PTX3 (and other disease parameters and markers).

To investigate a possible mechanistic connection to the inverse correlation between IFN- α and PTX3, we analyzed the influence of IFN- α on PTX3 release from PBMC and neutrophils (Figure 10). The production of PTX3 in PBMC was induced by IL-1 β and LPS, and to some extent also by TNF. The most distinct differences were seen after 24 h of stimulation. The PTX3 production induced by LPS was significantly inhibited by 54%, whereas IL-1 β -induced PTX3 production was significantly inhibited by IFN- α with a 78% median decrease. Also, the control PBMC was affected, implying that IFN- α inhibits the baseline synthesis of PTX3. We observed no reduced cell viability due to IFN- α exposure, hence the reduced PTX3 production was not due to a general decrease in cell viability/proliferation. Receptor blocking experiments showed that the suppression was mediated via the type I IFN receptor (IFNAR; Figure 10D).

Figure 10. Pentraxin 3 (PTX3) production from peripheral blood mononuclear cells (PBMC). The effect of interferon (IFN)-α on PTX3 production induced by interleukin (IL)-1β (20 ng/ml), tumor necrosis factor (TNF) (25 ng/ml) or lipopolysaccharide (LPS) (10 ng/ml) in PBMC stimulated for **A**) 3 h, **B**) 6 h and **C**) 24 h (n=6). **D**) Percentage inhibition of PTX3 by IFN-α. Effect of a neutralizing antibody to type I interferon receptor (IFNAR). PBMC were preincubated with 5 μg of antibody for 2 h and then stimulated with IFN-α and IL-1β (n=3). *=p<0.05.

In neutrophils, PTX3 release was induced by TNF and LPS, respectively, but not by IL-1β (Figure 11). On the contrary, the secretion was stimulated by IFN-α. The highest relative increase in PTX3 concentration in neutrophil supernatants was observed after 16 h of stimulation (34%) for TNF, whereas LPS induced an increase of 30% after 1.5 h. Neutrophils have been described as a reservoir of 'ready-to-use' PTX3 [55] and the PTX3-levels in cell culture supernatants were relatively stable over time, indicating a quick release/degranulation and not de novo synthesis.

Figure 11. Pentraxin 3 (PTX3) release from neutrophils. The effect of interferon (IFN)-α on PTX3 release induced by interleukin (IL)-1β (20 ng/ml), tumor necrosis factor (TNF) (25 ng/ml) or lipopolysaccharide (LPS) (10 ng/ml) in neutrophils stimulated for **A)** 1.5 h, **B)** 3 h, **C)** 6 h and **D)** 16 h, (n=6).

IFN-α exerted diverse effects on PTX3 and induced release from neutrophils and inhibited the synthesis in PBMCs. It is possible that IFN-α triggers tissue-recruited neutrophils to degranulate and deposit PTX3, in line with its suggested action in rheumatoid arthritis [102]. The net effect is likely reduced levels of PTX3 since SLE-patients with raised circulating IFN-α have reduced levels of circulating PTX3.

In a recent study, mice immunized with PTX3 developed anti-PTX3 antibodies which delayed lupus-like nephritis and prolonged survival [67]. The role of PTX3 in SLE and the handling of waste-disposal is far from clear. PTX3 is often referred to as a ‘double-edged sword’, since it has both pro- and anti-inflammatory properties. PTX3 can contribute to the waste disposal of cell debris and elimination of pathogens by opsonization [51]. Anti-inflammatory actions of PTX3 include that the binding to apoptotic cells can inhibit recognition by dendritic cells, and the ability to induce macrophage secretion of immunosuppressive cytokines, such as IL-10 and TGF-β. On the other hand, if the recognition by dendritic cells is inhibited, it is argued that

the removal of apoptotic cells would be hampered, rendering more cell debris left in the tissue, fueling the vicious inflammatory circle in SLE [103].

Future *in vitro* studies could include isolation of PBMC from SLE-patients and stimulate them to produce PTX3. Will the production be reduced relative to healthy controls?

PAPER III & IV

Osteopontin is associated with disease severity and antiphospholipid syndrome in well characterised Swedish cases of SLE

and

Osteopontin is a marker of disease activity, but not a distinct predictor of future damage in recent-onset systemic lupus erythematosus: Results from the Systemic Lupus International Collaborating Clinics inception cohort

OPN has been proposed as a marker of organ damage in pediatric lupus [74], and hence we wanted to investigate this protein as a potential marker of disease activity and/or organ damage in our KLURING cohort with adult SLE patients.

Serum OPN levels were in average raised fourfold in SLE cases compared to the controls. A weak correlation ($r=0.21$) was found between OPN and disease activity (i.e. SLEDAI-2K), which was more convincing specifically in patients with recent-onset disease ($r=0.67$). A correlation was also found between OPN and organ damage (SDI). Patients with extensive damage (i.e. $SDI \geq 3$) displayed increased levels of OPN compared to patients with moderate damage (i.e. SDI 1-2) and no damage (i.e. $SDI=0$) (Figure 12). Furthermore, separating SDI into different organ systems revealed a significant positive impact on OPN levels for the renal ($p<0.0001$), cardiovascular ($p<0.0001$), and malignancy domains ($p=0.012$). The SDI increase (the difference in SDI score at inclusion versus 2-6 years after inclusion) showed significantly higher OPN levels among patients with moderately or highly elevated SDI after study inclusion, compared to patients without SDI increase (Figure 12). This could imply that OPN is a marker of future organ damage. However, this may be biased by the fact that pre-existing organ damage *per se* predicts further subsequent organ damage [104-106].

Figure 12. Association between serum osteopontin (OPN) and damage accrual. **A)** Correlation between serum levels of OPN and Systemic Lupus International Collaborating Clinics/American College of Rheumatology damage index (SDI). Correlation coefficient and p value are not adjusted for sex, age, corticosteroid therapy and disease duration. **B)** Patients with extensive damage (i.e. SDI ≥ 3) displayed increased levels of OPN (mean 68.4 ng/ml) compared to patients with moderate damage (i.e. SDI 1-2; mean 36.0 ng/ml) and no damage (i.e. SDI=0; mean 35.6 ng/ml). **C)** Patients with highly elevated SDI (i.e. SDI increase 3-8; mean 62.9 ng/ml) and moderately elevated SDI (i.e. SDI increase 1-2; mean 50.4 ng/ml) had significantly higher OPN levels compared to patients with no SDI increase (mean 34.8 ng/ml). Crosses indicate the percentage of deceased patients for each SDI category.

Univariate general linear model showed that OPN levels were associated with APS ($p=0.009$) (Figure 13). When dissecting APS with regard to clinical manifestations related to APS, we found arterial event ($p=0.044$), myocardial infarction ($p=0.019$), ischemic stroke ($p=0.026$), arterial emboli ($p=0.031$), valvular heart disease ($p<0.0001$) and valvular surgery ($p<0.0001$) to have positive significant impact on OPN levels. Regarding laboratory items included in the APS criteria (i.e. the lupus anticoagulant, anti-cardiolipin and anti- β_2 glycoprotein-1 antibodies), we found associations with a positive lupus anticoagulant test ($p=0.033$) and IgM

anti-cardiolipin antibodies ($p=0.027$). To our knowledge this is the first study reporting a relationship between OPN and APS in SLE.

Figure 13. Serum osteopontin (OPN) levels in SLE cases with antiphospholipid syndrome (APS). Patients classified with APS showed higher levels of OPN (mean 54.1 ng/ml) compared to patients without APS (mean 38.2 ng/ml). The p value is adjusted for sex, age, corticosteroid therapy and disease duration.

OPN levels have previously been linked to manifestations on the arterial side, such as the severity of coronary atherosclerosis, increased risk for major adverse cardiac events and peripheral arterial disease [107, 108]. The role of OPN in cardiovascular disease is not clear. Some studies suggest OPN as a pro-inflammatory enhancer of atherosclerosis [107, 109], while other propose OPN as a protector in post-myocardial infarction by recruiting macrophages and neutrophils to clean-up dead cell debris [110].

Taken together our cross-sectional analyses imply OPN as a marker of disease activity at recent-onset disease, but later on in established disease it appears to reflect organ damage. The latter called for a longitudinal study targeting if levels of OPN are altered in the circulation prior to organ damage and thus an early predictor of future damage? Consequently, we analyzed OPN in a unique international multi-center cohort, the SLICC cohort, with 345 SLE-patients with recent-onset disease and 5-year follow-up data regarding disease activity and organ damage.

In line with our previous study from the KLURING cohort, serum OPN levels were in average raised fourfold in SLE-patients compared to controls. After 3 years, 19% ($n=64$) of the patients had developed damage (i.e. $SDI \geq 1$), and after 5 years this percentage was 29% ($n=99$). Weak correlations were found between OPN and accrual of organ damage after 3 ($r=0.112$, $p=0.037$) and 5 ($r=0.156$, $p=0.004$) years. Binary logistic regression was used to predict damage after 5 years, and to be able to adjust for age, sex, ethnicity and corticosteroid therapy. OPN levels

predicted damage ($p=0.024$), with an area under curve (AUC) of 0.65. However, when adjusting the cut-off for damage to $SDI \geq 2$, OPN levels did no longer predict damage. Notably, only 33 cases had an SDI of 2 or more. Stratifying patients based on SDI after 5 years into no damage (i.e. $SDI=0$), moderate damage (i.e. $SDI 1-2$) and extensive damage (i.e. $SDI \geq 3$) groups, revealed no significant differences. These results indicate OPN levels as a poor marker of future organ damage.

We also investigated OPN as a possible marker of disease activity. We found a correlation with disease activity at inclusion ($r=0.272$, $p<0.0001$). Patients with active disease, i.e. SLEDAI-2K value ≥ 5 , had higher levels of OPN (mean 56.8 ng/ml) than patients with in-active disease, i.e. SLEDAI-2K value <5 (mean 38.5 ng/ml), Figure 14.

Figure 14. Serum osteopontin (OPN) levels in patients with high and low/no disease activity. Patients with a SLEDAI-2K value ≥ 5 had higher levels of OPN (mean 56.8 ng/ml, $n=132$) than patients with SLEDAI-2K <5 (mean 38.5 ng/ml, $n=213$).

Moreover, we separated patients based on 'persistently raised disease activity' (i.e. $SLEDAI \geq 5$ on three separate occasions during 5 years). Higher levels of OPN were seen in the 52 patients with persistent disease activity (mean 53.7 ng/ml) compared to those without (mean 38.5 ng/ml), $p=0.0005$ (Figure 15). Associations remained significant after adjustments for age, sex, ethnicity and corticosteroid therapy in univariate general linear models. To investigate the importance of damage for OPN levels in the patients with persistently raised disease activity, we compared patients who had developed any damage (i.e. $SDI \geq 1$) after five years compared to those that did not develop damage. No significant differences in OPN levels were observed.

Figure 15. Serum osteopontin (OPN) levels in patients with persistently raised disease activity. **A)** Higher levels of OPN were found in the 52 patients with persistent disease activity (mean 53.7 ng/ml) compared to those without (mean 38.5 ng/ml, $n=293$). **B)** To investigate the possible impact of damage on OPN levels in cases with persistently raised disease activity, we compared patients who had developed any damage (i.e. $SDI \geq 1$) after 5 years to those without damage. No significant differences in OPN levels were observed between patients with any damage (mean 81.4 ng/ml, $n=19$) compared to those without (mean 51.4 ng/ml, $n=33$).

Binary logistic regression was also used to assess if OPN could predict persistently raised disease activity, and to be able to adjust for age, sex, ethnicity and corticosteroid therapy. OPN levels predicted persistently raised disease activity ($p=0.01$, $AUC=0.67$). OPN has earlier been suggested as a marker of disease activity in SLE [73], which is in line with our results.

Taken together our results from the SLICC cohort indicate OPN as a marker of disease activity rather than future damage. As mentioned before, only 33 cases had an $SDI \geq 2$. It would be interesting to analyze OPN in a larger group where the patients have more extensive organ damage, and preferably also an even longer duration of follow-up (>5 years).

There are some differences between the results from the cross-sectional KLURING study and the longitudinal SLICC study. The cross-sectional study implies OPN as a marker of disease activity predominantly in patients with recent-onset disease, and later on in established disease as a marker of organ damage. In contrast, the results from our longitudinal study suggests OPN as a marker of disease activity rather than organ damage. It is indeed possible that OPN could flag for organ damage at time of sampling, but likely not for prospective organ damage. It is also possible that the 5-year follow-up time in the longitudinal study is too short. One could argue that the two cohorts differ in ethnicity which could influence the results. It

is known that ethnicity can affect the severity of SLE and especially African-Americans experience the disease more severely than other racial-ethnic groups [111]. However, separating Caucasians and non-Caucasians in the two studies revealed different results. In the longitudinal study OPN levels among non-Caucasians correlated with organ damage, compared to the Caucasian group that lacked such correlation. Conversely, in the cross-sectional study the Caucasian group showed a correlation between OPN and organ damage that was not found in the non-Caucasian group. Notably, only 23 patients in the cross-sectional cohort were non-Caucasians.

Overexpression of OPN in lupus-prone mice induces B-cell activation and subsequent production of anti-dsDNA antibodies [76, 77]. However, we did not find any association with anti-dsDNA antibodies in the cross-sectional KLURING cohort. Intracellular expression of OPN in pDC has been reported to be required for TLR-9-dependent expression of IFN- α [78]. In addition, mutations in tartrate-resistant acid phosphatase (TRAP) cause spondyloenchondrodysplasia, an unusual recessive disease associated with short stature, brain calcifications and lupus-like autoimmunity [112]. OPN is a substrate for TRAP, and TRAP has been shown to co-localize and physically interact with OPN in pDCs and macrophages [113]. Lack of TRAP leads to hyperphosphorylation of OPN and enhanced TLR-9 signaling in pDCs with subsequent IFN- α production, which can cause the lupus-like autoimmunity seen in spondyloenchondrodysplasia patients. No association was found with IFN- α in the cross-sectional cohort. It would be interesting to measure IFN- α in the serum samples from the longitudinal study. As mentioned in the PTX3 study (Paper II), many patients with increased type I IFN signature lack measurable IFN- α in serum [98, 99]. Hence, it would of course be of high interest to measure the type I IFN signature and see whether it relates to OPN or not.

CONCLUSIONS

We have analyzed anti-HMGB1 antibodies, PTX3 and OPN in SLE patients and tried to relate the proteins to disease activity and/or organ damage. We have also investigated the relationship between PTX3 and IFN- α . The main findings are concluded below:

- Anti-HMGB1 autoantibodies occur in SLE and correlate with some disease activity variables. Anti-HMGB1 antibodies do not give rise to nuclear staining of the widely used HEp2-cell IF-ANA microscopy slides.
- Stimulation with IFN- α leads to release of PTX3 from neutrophils and attenuated synthesis of PTX3 by PBMCs *in vitro*. The net effect is likely reduced circulating levels of PTX3, since SLE patients with raised levels of IFN- α have lower amounts of PTX3 in serum.
- In the Swedish cross-sectional SLE cohort, circulating OPN correlates with disease activity in recent-onset disease, reflects global organ damage in more established disease, and associates with APS.
- In the international longitudinal multi-center study with recent-onset SLE cases, the performance of OPN regarding prediction of organ damage was not impressive. Circulating OPN associated with disease activity at disease onset, as well as over time.

A graphical summary of the thesis project is shown in Figure 16.

Figure 16. Graphical summary of the thesis project with potential interactions. Patients with SLE display an increased apoptosis rate in combination with an impaired clearance. The levels of anti-HMGB1 antibodies were elevated in SLE patients and correlated with some disease activity variables (Paper I). Decreased levels of PTX3 were found in SLE patients, especially in patients with raised circulating IFN- α (Paper II). PTX3 contributes to the waste disposal and low levels could thus result in accumulation of cell debris and subsequent inflammation in a vicious circle. SLE patients displayed elevated levels of OPN (Paper III & IV). Our results suggests OPN as a marker of disease activity in recent-onset disease, while it reflects organ damage in cases with more established disease. Green arrows indicate stimulation and red inhibition.

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