Studies on Cardiovascular Disease in Systemic Lupus Erythematosus

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Studies on cardiovascular disease in systemic lupus erythematosus

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Som med vederbörligt tillstånd av medicinska fakulteten vid Lunds Universitet, för avläggande av doktorsexamen i medicinsk vetenskap, kommer att offentligen försvaras i Reumatologiska klinikens föreläsningssal, Lottasalen Universitetssjukhuset i Lund. Fredagen den 24 november 2017, kl. 9.00

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Systemic lupus erythematosus (SLE) is a chronic, inflammatory, autoimmune multiorgan disease characterized by excessive production of type I interferons (IFNs) and autoantibodies against nucleic acids and an increased prevalence of cardiovascular disease (CVD). In this thesis, the connections between immunopathology in SLE and cardiovascular disease have been investigated.

Endothelial dysfunction (ED) is an early stage in atherosclerosis. Activation of the type I IFN system is central in SLE pathogenesis and has been shown to contribute to ED. Less is known about platelets in SLE, but platelets interact with both immune cells and endothelial cells.

In our current study, ED was seen in SLE patients with activated type I IFN system and platelet activation. Endothelial function was also assessed in relation to the vasculoprotective apolipoprotein M (apoM) since lipid dysregulation is often seen in SLE and apoM could be affected by inflammation. Lower plasma apoM levels in SLE patients were associated with disease activity and ongoing inflammation. In young patients, lower apoM correlated with ED.

Dysregulated neutrophils may release the pro-inflammatory protein complexes S100A8/A9 and S100A12 which through receptors on immune cells, might contribute to vascular damage in SLE. We found that serum levels of S100A8/A9 and S100A12 were increased in SLE patients with active disease, in patients with untreated glomerulonephritis, and in SLE patients with cardiovascular disease. Thus, inflammation caused by SLE could be related to involvement of neutrophils and the development of CVD.

In conclusion, elevated S100A8/A9 and S100A12 may indicate a role for neutrophils in CVD in SLE. Type I IFN activity might, together with activated platelets and apoM, affect the endothelium, leading to development of CVD in SLE. Our findings connect central pathogenic processes in SLE with CVD.

Key words SLE, Endothelial dysfunction, type I interferon system, S100A8/A9, S100A12

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Content

Abstract

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List of papers

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

I. Tydén H, Lood C. Gullstrand B, Jönsen A, Nived O, Sturfelt G, Truedsson L, Ivars F, Leanderson T and Bengtsson AA
Increased serum levels of S100A8/A9 and S100A12 are associated with cardiovascular disease in patients with inactive systemic lupus erythematosus. *Rheumatology (Oxford)*. 2013 Nov; 52 (11): 2048-55

II. Tydén H, Lood C, Gullstrand B, Jönsen A, Ivars F, Leanderson T and Bengtsson AA. Pro-inflammatory S100 proteins are associated with glomerulonephritis and anti-dsDNA antibodies in systemic lupus erythematosus. *Lupus* 2017 26(2), pp. 139-149.

III. Tydén H, Lood C, Gullstrand B, Nielsen C.T, Heegaard N.H.H, Kahn R, Jönsen A and Bengtsson AA. Endothelial dysfunction is associated with activation of the type I interferon system and platelets in patients with systemic lupus erythematosus *Accepted* for publication and a final version will be published in *RMD Open*

IV. Tydén H, Lood C, Jönsen A, Gullstrand B, Dahlbäck B and Bengtsson AA. Low plasma concentrations of Apolipoprotein M are associated with disease activity and endothelial dysfunction in systemic lupus erythematosus *Manuscript*

List of papers not included in the thesis

Lood C, Tydén H, Gullstrand G, Nielsen C.T, Heegaard N. H.H, Linge P, Jönsen A, Hesselstrand R, Kahn R, Bengtsson AA. Decreased platelet size is associated with platelet activation and anti-phospholipid syndrome in systemic lupus erythematosus. *Rheumatology (Oxford)*. 2017 Mars; 56 (3): 408-416

Lood C, Tydén H, Gullstrand B, Jönsen A, Källberg E, Mörgelin M, Kahn R, Gunnarsson I, Leanderson T, Ivars F, Svenungsson E, Bengtsson AA. Platelet-derived S100A8/A9 and cardiovascular disease in systemic lupus erythematosus *Arthritis Rheumatol*. 2016 Aug;68(8):1970-80.

Lood C, Tydén H, Gullstrand B, Klint C, Wenglén C, Nielsen CT, Heegaard NH, Jönsen A, Kahn R, Bengtsson AA. Type I interferon-mediated skewing of the serotonin synthesis is associated with severe disease in systemic lupus erythematosus. *PLoS One*. 2015 Apr 21;10(4)

Lood C, Tydén H, Gullstrand B, Sturfelt G, Jönsen A, Truedsson L, Bengtsson AA. Platelet activation and anti-phospholipid antibodies collaborate in the activation of the complement system on platelets in systemic lupus erythematosus *PLoS One*. 2014 Jun 12;9(6)

Abbreviations

ACR	American College of Rheumatology
AI	Augmentation index
AMI	Acute myocardial infarction
ANA	Anti-nuclear antibodies
Anti β2GP1	Anti-beta2 glykoprotein 1
Anti-CL	Anti-cardiolipin antibodies
Anti-dsDNA	Anti-double stranded DNA
aPL	Antiphospholipid antibodies
Apo M	Apolipoprotein M
APS	Antiphospholipid syndrome
BLyS	B lymphocyte stimulator
CRP	C-reactive protein
CVD	Cardiovascular disease
CVE	Cardiovascular event
CVI	Cerebrovascular incident
DAMP	Damage-associated molecular pattern
dsDNA	double stranded DNA
ED	Endothelial dysfunction
ELISA	Enzyme linked immunoassay
EMP	Endothelial microparticle
FcγR	Fc gamma receptor
FITC	Fluorescein isothiocyanate
HDL	High density lipoprotein
IC	Immune complex
ICAM-1	Inter Cellular Adhesion Molecule 1
IFN	Interferon
Ig	Immunoglobulin

IL	Interleukin
LDL	Low density lipoprotein
mDC	Myeloid dendritic cell
NCM	Necrotic cell material
PBMC	Peripheral blood mononuclear cell
pDC	Plasmacytoid dendritic cell
PMN	Polymorphonuclear leukocyte
PRR	Pattern recognition receptor
RAGE	Receptor for advanced glycation end products
RHI	Reactive hyperemia index
SLE	Systemic lupus erythematosus
SLEDAI	SLE disease activity index
SLICC/ACR-DI	Systemic lupus international Collaborating clinics/American College of Rheumatology damage index
sVCAM-1	Soluble Vascular Cell Adhesion Molecule 1
TLR	Toll like receptor

Introduction

Systemic lupus erythematosus (SLE) is a chronic, autoimmune disease with involvement of multiple organ systems. The disease usually affects women of childbearing age and is associated with severe comorbidity and premature mortality where cardiovascular disease is one of the more significant comorbidities occuring. The reasons for the elevated prevalence of cardiovascular disease seen in SLE need further investigation. This thesis comprises 4 studies of patients with SLE with emphasis on cardiovascular disease. The thesis investigates:

1. The role of the proinflammatory molecules S100A8/A9 and S100A12 in relation to cardiovascular disease, organ damage and disease activity (paper I and II).
2. Endothelial dysfunction in relation to type I interferon- and platelet activation (paper III).
3. Levels of the antiatherogenic apolipoprotein M in relation to disease activity and endothelial dysfunction (paper IV).

Systemic lupus erythematosus

Epidemiology

The incidence and prevalence of SLE have been investigated in a large number of epidemiological studies with varying results. These differences most likely dependent on different study design, methods to classify the patients, the ethnical background of patients investigated, exposure to a range of environmental factors and undiagnosed cases of SLE present in the studied population[1,2]. Furthermore, increasingly better diagnostic methods may affect the results. In two recent, large epidemiological studies from Michigan and Georgia in the United states, the incidence was consistent with 5.5-5.6 new SLE cases per 100 000 inhabitants[3,4] and a prevalence of 72.8 and 73.0/100 000, respectively during 2002-2004. In a study from the United Kingdom (UK), SLE incidence and prevalence were consistent during the years of observation 1999-2012: 4.9/100 000 and 65/100 000 1999 to 67/100 000 in 2012[5]. In a defined part of southern Sweden, all patients with SLE > 16 years of age, were identified between 1981-1991 by a validated capture-recapture method[6]. The incidence of SLE was relatively unchanged with 4.5-4.8 new cases per 100 000[7,8] . In a follow up study from the same region, SLE incidence declined to 2.8 new cases per 100 000 inhabitants between 1994 and 2006[9]. SLE is more common in certain ethnicities compared to Caucasians. In the United States, SLE is about 3 times more common in African American females compared to Caucasian females[4,10] and in studies from the UK a 5-9 fold increase of SLE incidence was found in Afro-Caribbean individuals compared to Caucasians[11,12]. SLE is more common in women than in men with a female/ male incidence ratio of 5-14/1, according to previous studies.[10,11,13]

Etiology and pathogenesis

Introduction

SLE is an autoimmune inflammatory disease involving several organ systems. Loss of immune tolerance and production of autoantibodies from hyperreactive B cells are central mechanisms behind development of SLE[14]. SLE patients have increased cell death, triggered by among other factors UV light, viruses or other environmental factors such as medication[15]. The cell remnants constitute an important autoantigen source and challenge immune tolerance in individuals with a genetic predisposition for autoimmune disease.

Genetics and environment

An interplay between genetic and environmental factors predispose a person to disease development in SLE. A concordance rate of 24% has been observed in monozygotic (MZ) and 2% in dizygotic twins, indicating the importance of genetics in the SLE etiology[16]. Mutations of certain genes increase the risk for disease development and variants of other genes are protective. Recent genome-wide association studies (GWAS) have made it possible to identify risk loci for SLE and so far over 40 risk genes have been defined[17]. A number of other autoimmune diseases share the same risk genes with SLE, for example haplotypes in the HLA region on chromosome 6[18]. Risk genes often code for proteins of importance in immune mechanisms, including the type I IFN system or T- and B cells[19,20].

Epigenetic modification of gene expression, through DNA methylation, modification of histones and micro-RNAs are suggested to contribute to development of autoimmune diseases including SLE[21]. Studies on discordant MZ twins have shown differences in DNA methylation between SLE- and healthy MZ twins in genes encoding immune functions[22].

A large number of environmental factors have been proposed to trigger SLE disease in individuals with genetic susceptibility including pharmacological agents[23], dietary factors, cigarette smoking and UV light. Viral infections such as Epstein-Barr virus (EBV), parvovirus 19 and endogenous retroviruses[24], can induce SLE disease by different mechanisms. IFN-α release during viral infection can trigger SLE disease development and disease exacerbation. Cross-reactivity between EBV and autoantigens was suggested to provoke SLE disease onset[25].

Immunopathology

The complement system

The complement system is an important part of host defense and constitutes a link between the innate and adaptive immune responses. Its major functions are:

- Host defense against invading pathogens
- Clearance of immune complexes and dying cells
- Augmentation of the adaptive immune system

The complement system consists of a large range of plasma- and membrane-bound proteins[26], most of them produced in the liver. Once the first protein is activated, a chain reaction is started where one component activates the next. This cascade reaction leads to the formation of C3 and C5 convertases. During activation, split products are generated which are antimicrobial, opsonins and chemoattractants and mediate inflammation by recruitment and activation of immune cells. Eventually the membrane attack complex (MAC) is formed.

The cascade reaction is closely regulated to avoid uncontrolled activation and tissue damage. Different regulatory proteins are present in many of the steps in the cascade[27].

Three different pathways of complement activation are described: the classical pathway, that plays an important role in SLE pathogenesis, the lectin pathway and the alternative pathway (figure 1).

The classical pathway

The components involved in the classical pathway are C1, C4 and C2. The classical pathway is activated by C1q binding to immune complexes (IC) or apoptotic cells. When C1q, produced by dendritic cells and macrophages, bind to IC, a conformational change of the C1 complex occurs and C1r and C1s becomes activated. The activated $C1qr_2s_2$ enzymatically cleaves C4 and C2, leading to formation of C3 convertase (C4b2a) capable of cleaving C3[28].

The lectin pathway

The lectin pathway is activated when carbohydrates *e.g.*mannose on the surface of bacteria bind mannose binding lectin (MBL)[29] in the presence of MBL-associated serine proteases (MASPs). MASPs cleave C2 and C4 with subsequent formation of C3 convertase (C4b2a)[30]. At this step, the lectin pathway converges with the classical pathway.

The alternative pathway

C3 is spontaneously hydrolyzed in the presence of Mg^{2+} . C3 interacts with factor B, allowing factor B to be cleaved by factor D, leading to formation of an initial C3 convertase, C3(H2O)Bb, capable of cleaving C3 to C3a and C3b. C3b generated from either pathway, binds to factor B and after cleavage by factor D, C3 convertase of the alternative pathway (C3bBb) is formed[31].

The terminal pathway

All three pathways lead to cleavage of C3 and the formation of C5 convertase which initiate the common, terminal pathway. C5b bind to C6 and C7[32] with subsequent C8 binding resulting in the formation of the C5b-8 complex. Multimers of C9 are bound to the C5b-8 complex with formation of the membrane attack complex (MAC), that cause cell lysis and cell death[26,27,33].

Function of the complement system

Besides defense against pathogens, the complement system is important for clearance of apoptotic and necrotic cell material, as well as IC handling, as mentioned above. The complement components, mark apoptotic cell debris and ICs so called opsonization and facilitates their phagocytosis by immune cells and their eventual clearance. The complement system also has a function during the development of the immune system for elimination of self-reactive lymphocytes. Self-antigens covered with complement fragments, bind to B cells by complement receptor 1 (CR1) or CR2, with subsequent clearance, a mechanism to maintain tolerance.

The complement system in SLE

The elimination of autoreactive B lymphocytes is impaired in SLE due to complement deficiency. Complement deficiency in SLE is caused by consumption of components by activation of the pathway or through a hereditary deficiency. The importance of the complement system in defense against pathogens and in sustained tolerance is demonstrated in patients with hereditary classical complement deficiencies; C1q, C4 or C2[34]. These patients have an increased risk for serious infections by certain bacteria[35] and an elevated risk of developing SLE[36]. In fact, nearly 100% of known individuals with C1q deficiency develop SLE. Though a very rare condition, it indicates the importance of C1q to maintain health. If immune complex deposit in tissue, subsequent complement deposition and neutrophil infiltration can lead to an inflammatory reactions resulting in tissue damage. This is seen in SLE glomerulonephritis[34,37]. In all, the complement system protects us from invading pathogens and furthermore protects against inefficient clearance of cell debris, thus decreasing the risk for autoimmunity. On the other hand, its activation can cause tissue damage and in this way be harmful to us.

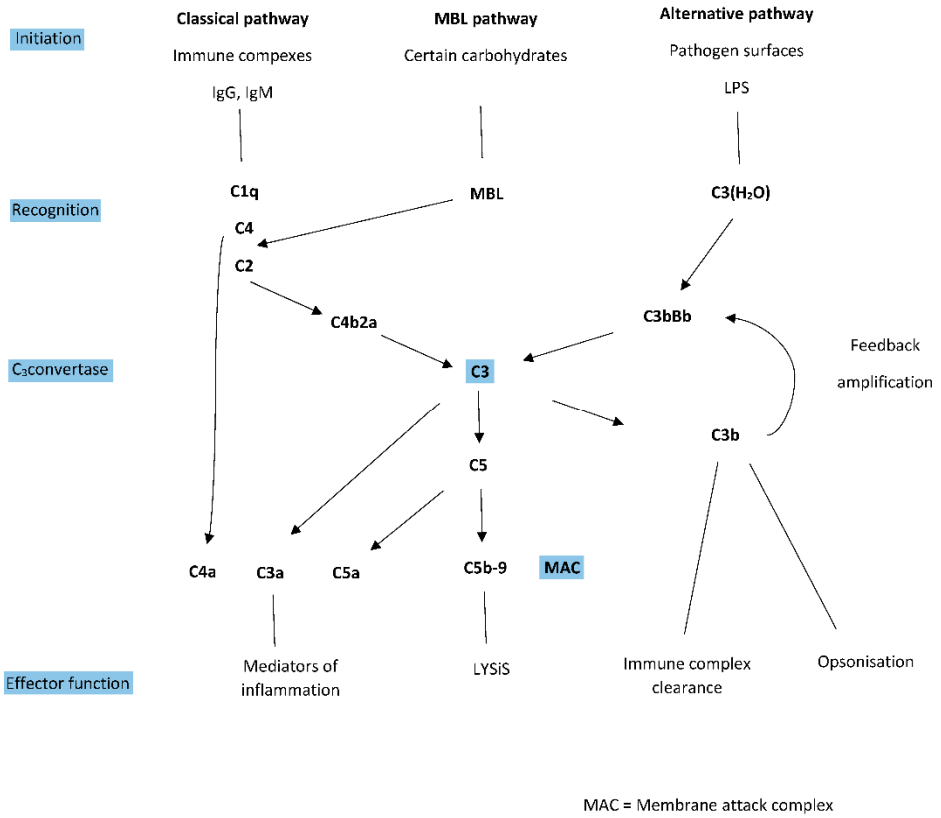


Figure 1. Simplified picture of the complement system

Clearance of apoptotic material and immune complex

Under normal physiological conditions, cell death is controlled by a strictly regulated mechanism called apoptosis, programmed cell death. The apoptosis process is fast and "silent", without inflammation. The dying cells release so called "find me" signals that are recognized by phagocytes such as macrophages. The dying cell also expose "eat me" signals on the cell surface. Receptors on the surface of the phagocytes recognize the apoptotic cell which is then rapidly cleared[38,39]. Under non-pathological conditions the membranes of the organelles in the shrinking, apoptotic cells are still intact when they are phagocytosed, thus inflammation is not induced. Different molecules including components of the classical pathway in the complement system; C1q and C3, can act as bridging molecules between the dying cell and the phagocytes. The complement components can also bind directly to the cell surface, and facilitate its clearance by opsonization and an anti-inflammatory response is started[40]. After phagocytosis of apoptotic material, macrophages produce anti-inflammatory cytokines: TGF β and

IL-10, inducing immunosuppressive effects to prevent inflammation[40]. Milk fat globule EGF factor 8 (MFG-E8) is a soluble protein that binds to apoptotic cells as an opsonin and facilitates removal by phagocytes. MFG-E8 also affects the intracellular processing of the apoptotic material, and inhibits the phagocyte, e.g. the macrophage, presenting the autoantigens on their surface. Indeed, MGF-E8 deficient mice develop lupus-like disease, indicating a role for opsonins in preventing autoimmune disease[41].

While cell death by apoptosis is a strictly regulated, non-inflammatory process, cell death by necrosis is caused by harmful exposures such as burn injury, severe infection/sepsis or radiation. The damaged cells rapidly loose membrane integrity, resulting in influx of water and sodium into the cells that burst and expose their intracellular and nuclear contents to the immune system. The exposed antigens together with toxic enzymes and proteases from the necrotic cells result in a massive inflammatory response and tissue damage[42].

Secondary necrosis may also occur as a final step in the apoptosis process. If not cleared, some of the apoptotic cells may undergo secondary necrosis where plasma membrane integrity is lost and intracellular and nuclear antigens are exposed, a condition that is pro-inflammatory and might trigger an immune response[15].

Neutrophils may undergo another kind of cell death; NETosis. Neutrophil extracellular traps (NETs) is a mechanism for activated polymorhonuclear neutrophils (PMNs) to catch and destroy fungi and bacteria by releasing a net containing chromatin and microbicidal enzymes[42]. Besides apoptosis, necrosis and NETosis, other mechanisms for cell death exist, that will not be discussed in this thesis.

In addition to debris from apoptotic cells, nuclear antigens from cell necrosis or NETosis are also exposed to the immune system. The classical complement pathway is activated by ICs, C3b opsonize the IC and bind to complement receptor 1 (CR1) on erythrocytes. The erythrocytes with ICs are transported to the spleen and liver where the Fc portion of the IgG is bound to Fcγ receptors on the macrophages and the erythrocytes with ICs are phagocytized. In this way the nuclearantigen-containing ICs are cleared from the circulation.

Clearance of apoptotic material and immune complex in SLE

In SLE, there are several mechanisms contributing to an increased load of dying cells. As a result of an unknown “apoptosis factor” in SLE patients, apoptosis is increased, contributing to the larger amount of nuclear antigen-containing cell debris in the circulation, that challenge the immune system[43,44]. The large amount of cell remnants also derive from necrotic cells and NETting neutrophils. In SLE, clearance of this cell debris is defect and the cell remnants are thought to be a main source of autoantigens. Proposed mechanisms of defect clearance are 1).

decreased levels of components of the classical pathway in the complement system caused by consumption or hereditary deficiency[45] 2). Erythrocytes in SLE patients have a decreased expression of CR1 because of inherited deficiency or consumption through frequent IC/C1R interaction[46] 3). The macrophages in SLE patients have decreased capacity to clear apoptotic cell remnants[47]. This contribute to defect IC-erythrocyte clearance[34]. Furthermore, impaired function of FcγR on phagocytes, leads to defect clearance[48].

Thus, defect handling of IC and nuclear cell debris drive the autoimmune process in SLE.

Autoantibodies

Autoantibodies, specific to nucleic acid or to proteins associated with nucleic acids such as histones, are produced by hyperactive plasma cells and are key players in the SLE pathogenesis[14].

Anti-dsDNA antibodies have a high specificity for SLE and are part of the SLE classification criteria[49]. Dying cells are a major source of extracellular DNA, with the DNA usually being associated with histones, in structures known as nucleosomes[50]. Anti-dsDNA antibodies in SLE target DNA and, to a large extent nucleosomes[51]. Elevated anti-dsDNA antibodies as well as antibodies to C1q, can precede disease flare and are associated with active glomerulonephritis[34,52,53].

Anti-Sm, with high specificity for SLE[54], and anti-RNP antibodies target RNA-autoantigens.

Antibodies to SSA and SSB are associated with skin manifestations, Sjögren's syndrome and, though not often seen, maternal SSA/SSB antibodies can cause fetal cardiac atrio-ventricular block[55].

In addition to the ANAs, several other antibodies are described in SLE including antibodies to C reactive protein (CRP)[56] phospholipids and phospholipid- related proteins, erythrocytes and high density lipoprotein (HDL)[53,57].

Autoantibodies can be detected several years before onset of clinical SLE disease. In a study of prospectively collected blood samples from the United States military of which 130 individuals developed SLE, 88% were positive for at least one SLE-specific autoantibody years before clinical disease onset. The autoantibodies appeared in a predictable order with anti-Ro (SSA), anti- La (SSB) and aPL antibodies found earlier and anti-dsDNA, anti-Sm and anti-RNP were found closer to clinical disease onset[58].

To conclude, autoantibody production by hyperactive plasma cells is a hallmark of SLE. The autoantibodies may be pathogenic in SLE, can serve as diagnostic

markers, indicate certain clinical phenotypes and be used to follow disease activity.

CRP

CRP is a pattern recognition molecule of the innate immune system with potential protective or damaging effects depending on the local environment. Through binding to apoptotic cell material, CRP can facilitate their clearance by complement activation or by binding to Fcγ-receptor II on phagocytes, both leading to increased phagocytosis[59]. CRP is suggested to have anti-inflammatory properties when interacting with the complement components. Antibodies to CRP have been demonstrated in SLE and correlate with disease activity[56]. Thus, CRP might have protective effects in SLE. On the other hand, CRP is an acute phase protein, up-regulated by IL-6, having pro-inflammatory effects. CRP is also affected by IL-1β[60]. Furthermore, CRP has been reported to have harmful effects by playing a role in atherosclerosis development[61,62]. Thus, CRP has a variety of roles in immune modulation.

Immune cells

B cell abnormalities are seen in SLE with hyperactive B cells, leading to plasma cell-mediated production of autoantibodies and presence of memory B cells reactive to autoantigens[14]. A defective central tolerance mechanisms in early B cell development, increased numbers of B lymphocytes expressing auto-reactive B cell receptors (BCR) are seen in SLE[63]. Generation of autoreactive B cells in the periphery as a result of somatic hypermutation has been described[64].

In SLE, excessive amounts of dead cell material that may undergo changes when not cleared, contribute to an abundance of autoantigens.Increased numbers of plasma cells are demonstrated in SLE and levels correlate with titers of anti-dsDNA antibodies[65].

B cells are also suggested to stimulate disease progression in the absence of antibodies as they produce cytokines and are also APCs. Self-reactive B cells present autoantigens to T cells with subsequent activation and amplification of autoreactive T cell clones, promoting autoimmunity[14,66,67].

BLyS

B-Lymphocyte stimulator (BLyS) stimulates B cell differentiation to plasma cells, antibody production and promotes B cell survival[68,69]. BLyS production is stimulated by type I IFNs and serum levels increase with disease activity in SLE[70]. Furthermore, BLyS can promote rescue of autoreactive B cells that lead to break of tolerance[71]. Treatment with the BLyS blocker, belimumab, was reported to be effective for reduction of disease activity and serological activity[72,73].

To summarize, B cell homeostasis is disturbed in SLE. Increased B cell activation and autoreactive B cells that escape deletion by different mechanisms, eventually lead to plasma cell-mediated autoantibody production that is central in SLE pathogenesis.

T cell dysregulation in SLE

Hyperactivation of T cells is involved in the disease mechanism in SLE[74] and different factors contribute to enhanced T cell activation. Defective signaling through the T cells receptor (TCR) contributes to hyperreactivity[75]. Furthermore, abnormal B- and T cell interaction is seen[76]. Alterations of T cell subpopulations, in part by changes in cytokine profile, and impaired T cell tolerance is seen in SLE and contribute to the pathogenesis.

Polymorphonuclear leukocytes

Abnormal functions and changed phenotype of neutrophils are found in SLE and contribute to disease activity and organ damage.

Neutrophil granulocytes or polymorphonuclear leukocytes (PMNs) are cells of the innate immune system, abundant in humans with major roles in early defense against invading pathogens. PMNs have a short lifespan of a few hours to three days and new PMNs are continuously produced in the bone marrow where they differentiate before release. The circulating neutrophils adhere to the activated endothelial cells of the vascular wall and migrate into tissue towards inflammatory sites where they eliminate invading microbes through different mechanisms[77].

Functions of PMNs and their dysregulation in SLE

A major function of neutrophils is to engulf and destroy microbes by phagocytosis. The impaired phagocytosis reported in SLE might contribute to the elevated risk for infections seen in these patients[78].

Toxic substances capable of destroying microbes are released from PMN cytoplasmatic granules at inflammatory sites to eliminate pathogens. In addition, inflammatory mediators affecting immune cells are released from the granules[79].

The pro-inflammatory protein complex S100A8/A9 is the most abundant cytosolic molecule in neutrophils. When passively or actively secreted to the extracellular space, S100A8/A9 and S100A12, the calgranulins, act as DAMPs and exert their pro-inflammatory effects through pattern recognition receptors. The calgranulins play a potential role in the pathogenesis of inflammatory rheumatic diseases[80] and cardiovascular disease (CVD)[81] and this will be further discussed below in the section about S100 proteins.

NETs are a mechanism for neutrophils to trap and destroy pathogens and the process can be induced by different stimuli including IC and cytokines[82]. SLE

patients have decreased ability to degrade NETs, and this may be associated with elevated disease activity[83]. NETs that are not degraded and cleared may be an antigen source in SLE. NETosis lead to exposure of genomic and mitochondrial DNA to the extracellular (e.c.) environment. Reactive oxygen species (ROS), generated from activated neutrophils oxidize the e.c. DNA, making it pro-inflammatory[84].

Low density granulocytes (LDGs) are a subset of abnormal granulocytes found in SLE patients, with decreased capacity to phagocytose microbes, but increased ability to undergo NETosis, suggested to be driven by mitochondrial ROS production[84-86]. Oxidized mitochondrial DNA from LDG NETs were demonstrated to induce type I IFN production through TLR and/or a STING- dependent pathways[84], further described in the section about type I interferons below, thus this may contribute to the IFN signature in SLE patients.

The nuclei of LDGs have been analyzed and were demonstrated to be more segmented compared to neutrophils with normal density, indicating a more immature phenotype in this subset of neutrophils. Studies of the gene expression profile, also suggested an immature phenotype of LDGs[85]. LDGs have a pro-inflammatory phenotype with elevated secretion of several cytokines compared to normal density neutrophils. Increased secretion of TNF α, IL-6 and IFN α have been demonstrated and might play a role in different disease manifestations and organ damage development in SLE[87].

Neutrophil dysregulation in SLE and their effects on organ manifestations and damage

Neutrophils have been implicated as a contributing factor in SLE organ manifestations and organ damage by different mechanisms. This section will focus on the LE cell, glomerulonephritis and cardiovascular disease (CVD).

The LE cell

The LE cell is a neutrophil abnormality, seen is SLE, and was first described by Hargraves in 1948[88]. The LE cell was the first laboratory test used in diagnosis of SLE and it was previously included in the ACR classification criteria for the disease[89]. LE cells are polymorphonuclear neutrophils (PMNs) engulfing antibody- and complement-opsonized nuclear material. The engulfed material has given the LE cell its characteristic form with the nucleus displaced in the cell and can be visualized by light microscopy[90,91]. The LE cell test is rarely used in clinical praxis anymore, but it is described to correlate with clinically and serologically active disease and indicates a more severe disease[92]. In 2004 a new method to assess and quantify LE cells by flow cytometry was described[93].

Glomerulonephritis

A potential role for PMNs in SLE glomerulonephritis (GN) was suggested already in the 1980's when ICs bound to receptors on PMN membranes were found in kidney biopsy from SLE patients with GN[94]. Impaired clearance of NETs was associated with glomerulonephritis in SLE[95] and NET-producing neutrophils were found in kidney of SLE patients with renal involvement[86], which may indicate a role of NETting neutrophils in GN. In addition, elevated serum levels of S100A8/A9 have been demonstrated in SLE patients with untreated GN[96], (paper II), and might play a role in GN pathogenesis through receptor interaction, but this has to be further investigated.

Cardiovascular disease

LDGs are known to produce type I IFNs and also stimulate plasmacytoid dendritic cells (pDCs) to further IFN α production (se below). Type I IFNs have unfavorable effects on endothelial cells and also on endothelial repair, and in this way, contribute to atherosclerosis and CVD in SLE patients. LDGs have enhanced capacity to undergo spontaneous NET formation. NETs have cytotoxic effects on endothelial cells and could thus contribute to endothelial damage[86,87].

PMNs are the main sources of the S100A8/A9 and S100A12 suggested to be markers of CVD in SLE[81,97]. Furthermore, the calgranulins might play a role in atherosclerosis development[81,97], indicating another role for PMNs in CVD development in SLE patients.

To summarize, dysregulated neutrophils are mediators in the SLE pathogenesis and are implicated in severe organ manifestations such as renal involvement and organ damage including CVD. Further investigation of these cells is needed to find new biomarkers, predictors and targets for treatment in SLE.

Plasmacytoid dendritic cells (pDCs)

pDCs constitute less than one percent of PBMCs in human blood but play an important role in defense against viral infections. pDCs are immune modulating cells that affect other immune cells through the cytokines they produce. They are the main producers of IFN α, with immediate and high type I IFN production as a response to viral infection[98]. In SLE, pDCs are stimulated by IC to produce IFN α, described in detail in the section below about type I IFNs. In addition, pDCs produce the pro-inflammatory cytokines IL-6 and TNF-α when activated by viruses, as well as other chemokines that attract immune cells to sites of inflammation[99].

The interferons

Interferons (IFNs) have immune modulatory effects with important functions in host defense. The type I IFNs can restrain virus replication and in this way protect cells from viral infection [100]. Three groups of IFNs exist: type I (IFNα, -β, -ω, -κ, -ε), type II (IFNγ) and type III (IFNλ). Type I IFNs bind to the type I IFN receptors (IFNAR) that are expressed on nucleated cells[101]. IFNα is a key cytokine in SLE disease mechanisms and will be discussed below.

Initiation of type I IFN production

IFN α is produced mainly by immature pDCs in response to viral infection[98,102,103]. When activated, immature pDCs obtain a mature cell phenotype and lose their ability to produce IFN α[104].

Pathogens (viruses or bacteria containing RNA and/or DNA) ligate to Fcγ receptor IIa (FcγRIIa) on the surface of pDCs and are phagocytosed by a receptor-mediated endocytosis with subsequent activation of the pDC and production of type I IFNs[105]. Pattern recognition receptors (PRRs) located in the cytosole or endosome of the pDCs, recognize microbial nucleic acid from viruses or bacteria. The PRRs TLR 7 and TLR 9 are expressed on the endosomal surface in pDCs and recognize RNA and DNA, respectively. The TLR-nucleic acid interaction, that takes place in the endosome, leads to pDC activation and IFN α secretion[105,106].

In autoimmune rheumatic diseases such as SLE, pDCs can also be stimulated to produce IFN α by endogenous inducers,[107] e.g. DNA- or RNA-containing remnants from apoptotic and necrotic cell material that formed ICs with autoantibodies. Through FcγRIIa- mediated endocytosis in pDCs, RNA or DNA fragments are recognized by endosomal TLR 7 and 9 to trigger pDC activation and subsequent IFN α production. In SLE, with defect clearance of apoptotic and necrotic cell material, abundance of circulating nucleic acid-containing ICs might contribute to high serum levels of IFN α.

Recently, non-TLR-dependent pathways of type I IFN induction have been described. Cytoplasmic nucleic acid sensors such as RNA helicase retinoic acid-inducible gene 1-like receptors and DNA receptors are examples of such IFN α inducers[108,109]. It is known for a long time that cytosolic DNA, derived from nucleus or mitochondria of eukaryotic cells, is immunogenic. The cytosolic DNA sensor cyclic GMP-AMP synthase (cGAS) binds to cytosolic dsDNA, resulting in synthesis of cyclic GAMP (cGAMP). cGAMP act as a second messenger and binds to the cellular protein STimulator of Interferon Genes (STING) with subsequent activation of the transcription factor IRF 3 that induce synthesis of IFN β[110-112].

Defective clearance of cytosolic DNA can be due to deficient or dysfunctional DNases resulting in elevated type I IFN signaling and autoimmunity. Mutations of DNase I or DNase I deficiency, lead to disease with lupus-like features in humans and mice[113]. Further, mice deficient in DNase II, demonstrate accumulation of cytosolic, non degraded DNA, leading to enhanced type I IFN production with mice embryos dying in utero[114]. Mutation of the DNase III (Trex1) gene in humans is associated with SLE and the unusual, so called interferonopathy Aicardi-Goutières syndrome (AGS)[115,116]. In an animal model, mice with Trex 1 deficiency developed severe, IFN-mediated inflammation with presence of autoantibodies and autoimmune symptoms. These findings indicate a role for Trex 1 in degradation of intrinsic self DNA or DNA derived from phagocytosed dying cells[117].

Thus, impaired degradation of DNA may lead to activation of the DNA sensor cGAS, leading to a non-TLR-dependent increase in IFN production via the STING pathway.

Effects of IFN α

As mentioned above, IFN α can interfere with viral replication and induce apoptosis of cells infected with viruses[118]. IFN α has various effects on the immune system with a cumulative impact of an increased immune responses towards pathogens. Differentiation of B cells to plasma cells and increased production of antibodies is stimulated by IFN α[119]. Inhibition of apoptosis of activated T cells is mediated by IFN α, as well as enhanced activation of cytotoxic T cells[120,121]. Furthermore, IFN α enhances T helper cells to Th1 subset development[122]. IFN α enhances monocyte differentiation to antigen presenting cells, as well as maturation of dendritic cells[123]. The cytotoxic effect of NK cells is also promoted by IFN α[124].

Type I interferonopathies

A rare group of disorders with a persistent upregulated type I IFN response and SLE-related features has been described in recent years[125,126]. These interferonopathies are due to mutations in genes that possibly lead to accumulation of endogenous nucleic acid, where mutation in TREX1 is one example. Mutations in genes coding for proteins involved in nucleic acid-receptor interaction with subsequent IFN induction, such as STING, has also been described. Predominantly the pathways affected impact on induction of IFN-β, indicating a role for IFN β in the SLE pathogenesis[125,126].

IFNα in SLE

The important roles of IFN α in host defense have been discussed above. However, if not properly regulated and inhibited, an excessive IFN α-initiated

immune response can contribute to development of autoimmune disease. IFN-α and -β are used in cancer treatment, as the type I IFNs induce cell-death and also inhibit angiogenesis, that is important for tumor growth[127,128].The antiviral functions of type I IFNs have also been used in treatment of hepatitis C[129]. Patients with certain forms of cancer, hepatitis and multiple sclerosis (MS), treated with IFN-α or-β, have been observed to develop autoimmune, SLE-like disease[129-131]. The type I IFNs are suggested to contribute to break of tolerance by effects on immune cells, mentioned above.

An increased expression of type I IFN regulated genes, the so called IFN signature, is seen in SLE[132,133]. Furthermore, SLE patients have elevated serum levels of IFN α compared to healthy controls and serum levels correlate with disease activity[134,135]. These findings indicate an important role of type I IFNs in SLE disease mechanisms.

ICs containing DNA or RNA induce IFN α production from pDCs in SLE. Patients with SLE have low numbers of pDCs in peripheral blood that might be due to pDCs migration to tissue including lymph nodes, skin and kidney[136].

Part of the elevated type I IFN levels seen is SLE is suggested to be due to activation of the cGAS/STING pathway, since increased cGAS expression has been demonstrated in SLE patients, and a subset of patients had elevated cGAMP levels, most pronounced in patients with high disease activity[137].

Interaction between type I IFN and the endothelium in SLE and type I IFNs inhibitory effects on angiogenesis will be discussed in the section on cardiovascular disease in SLE.

Anti-IFN α receptor antibodies

Therapies directed against the interferon alpha receptor (IFNAR) are now developed and under evaluation. Anifrolumab is a monoclonal anti-IFNAR antibody. In a double-blind, randomized, placebo controlled phase IIb trial, SLE patients with moderate-to high disease activity were randomized to anifrolumab or placebo together with conventional therapy. Patients who received IFNAR blocker, achieved the primary end point of improvement in SLE responder index (SRI), more often than placebo with a good tolerability[138]. In a recent, double-blind, randomized, placebo controlled phase IIb trial, an anti-IFN α monoclonal antibody demonstrated efficacy with decreased disease activity in SLE patients with moderate to high disease activity with acceptable tolerability[139]. Future therapies affecting type I IFN levels are developing. Depletion of pDCs in lupus prone mice in vivo, resulted in decreased transcription in tissue of type I IFN-inducible genes and reduced activation of immune cells as well as decreased levels of anti-nuclear autoantibody production. Furthermore, renal pathology was

improved[140]. These findings indicate that pDC depletion might be a therapeutic opportunity in the future.

Regulation of IFN α production

In healthy individuals, the type I IFN production is strictly regulated. Several cell types have been shown to either amplify or suppress pDC-mediated IFNα production upon activation by ICs. Amongst the activating cells T cells, B cells, NK cells and platelets have been found. Although the exact mechanisms have not been fully elucidated, T cells are thought to stimulate IFN production through GM-CSF, whereas platelets act through the CD154-CD40 interaction[141-144].

Inhibitory regulation of IFN α production is impaired in SLE. Monocytes have an inhibitory effect on IFN α production in pDCs, mediated by reactive oxygen species, prostaglandin E2 and TNF-α[145]. The complement component C1q has been demonstrated to have regulatory effects on cytokine production, with the ability to inhibit IFN α production by pDCs. Lower C1q levels in SLE, due to consumption or presence of C1q autoantibodies was suggested to contribute to the defect inhibition of type I IFN activity in SLE[146].

Thus, type I IFNs are potent mediators of host defense against viral infection. Enhanced type I IFN signaling by various mechanisms might lead to diseases including SLE, where the aberrant IFN production affects immune cells and promotes autoimmunity.

To summarize, exposure to environmental factors in genetically susceptible individuals act together in the many different mechanisms that are proposed in the etiology and pathogenesis of SLE, contributing to breakdown of immunological tolerance. Cell remnants from apoptotic, necrotic and NETting cells, containing intracellular and nuclear material, are left in the tissue or in the circulation for prolonged periods of time due to defective clearance and challenged immune tolerance. Defective clearance of dying cells in SLE is partly due to lowered complement levels and impaired complement function[45], but several other mechanisms contribute to impaired clearance of necrotic and apoptotic cell debris in SLE. Autoreactive B lymphocytes produce autoantibodies and ICs are formed, that may deposit in tissue, activate complement and cause neutrophil infiltration. Inflammation can then be initiated, leading to organ manifestations, tissue destruction and, if not treated, organ damage[37,147]. IC-stimulated pDCs produce type I IFNs with stimulating effects on immune cells[105]. Furthermore, ICs might activate PMNs to release the pro-inflammatory proteins S100A8/A9 and S100A12. In the extracellular space they act as damage- associated molecular patterns (DAMPs) through pattern recognition receptors (PRRs) on inflammatory cells and mediate inflammation contributing to an inflammatory milieu[148]. Activated PMNs might release NETs containing nuclear materials and intracellular

proteins, including S100 proteins, and this would be another autoantigen source[149]. A vicious circle of autoimmunity and inflammation is set up leading to SLE disease (figure 2).

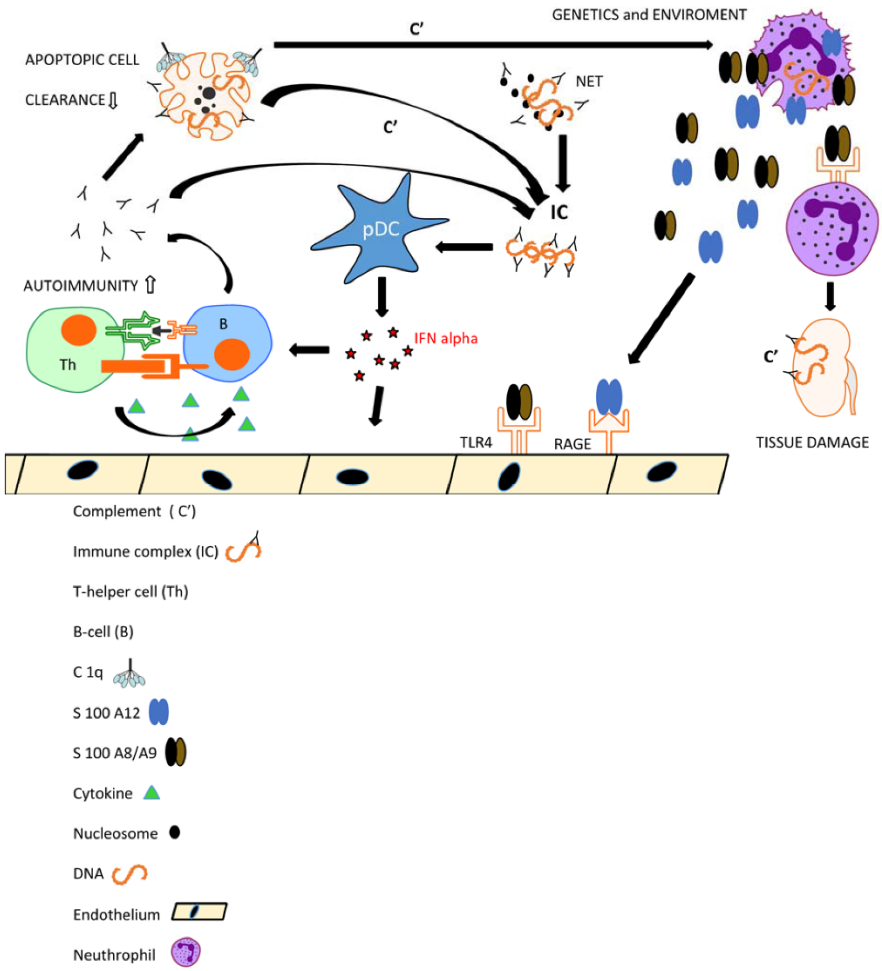


Figure 2 The pathogenesis in SLE
Partially based on biodraw pictures

Clinical features

Symptoms

The SLE disease course is characterized by periods of disease exacerbation (flares) and remission. There are different phenotypes of the disease, which can involve basically any organ system. The disease course can be mild, with symptoms

involving the skin and musculoskeletal system as the most prominent features, or severe with inflammation of vital inner organs including kidneys and the central nervous system (CNS).

General symptoms

Malaise, fever and fatigue can lead to reduced working ability and lower quality of life even if most often not signs of severe disease.

Musculoskeletal symptoms

Inflammation in joints with myalgia, migrating arthralgia, arthritis and tenosynovitis in small, peripheral joints affect most SLE patients and is often an early sign of disease. Chronic tenosynovitis can lead to hand deformities without cartilage or bone damage, these are known as Jaccoud deformities[150].

Skin and mucosa

Acute, subacute or chronic manifestations from the skin and mucosa are seen in about 70% of the patients and in many patients are the initial symptoms of disease. Photosensitivity, subacute cutaneous lupus, butterfly rash or discoid lesions as well as alopecia, oral and nasal mucous ulcers are also seen.

Cardiac manifestations

Pericarditis or perimyocarditis is seen in about 1/5 of patients. More seldom, Libmann-Sacks endocarditis is found and associated with presence of anticardiolipin antibodies (aCL). Endocarditis constitutes a risk for cerebral emboli[151]. The elevated risk for atherosclerosis and coronary artery disease will be discussed in detail in the section on cardiovascular disease

Pulmonary manifestations

The most common pulmonary manifestation is pleuritis, affecting about 1/2 of the SLE patients. Acute pneumonitis and interstitial lung disease (ILD) are uncommon, severe manifestations[152].

Haematological abnormalities

Leuko-lympho-and thrombocytopenia are seen in active disease and can be due to impaired production because of bone marrow inhibition or peripheral destruction by autoantibodies[153-155]. Secondary anemia is commonly seen while autoimmune hemolytic anemia is rarely described. It can be difficult to decide whether leuko- and thrombocytopenia is caused by disease activity or therapy, such as methotrexate or azathioprine.

Glomerulonephritis

Glomerulonephritis (GN) is a severe and common manifestation of SLE. Proteinuria, cellular casts in urinary sediment, low levels of complement components C3, C4 or C1q and presence of anti-dsDNA antibodies indicate GN. Kidney biopsy is recommended for diagnosis and classification of the GN according to the International Society of Nephrology/Renal Pathology Society (ISN/RPS) classification[156]. Immunosuppressive therapy is indicated for proliferative GN (class III-IV) and for membranous GN class V, described in the therapy section below. When response to treatment is evaluated, renal function, level of proteinuria and signs of activity in urinary sediment are estimated. Early detection of GN is crucial to avoid organ damage.

Nervous system

The central and peripheral nervous system can be involved in SLE and range from mild manifestations to severe. Headache, depression, minor cognitive impairment and fatigue are considered less serious and reported from the majority of SLE patients during some period of the disease course.

Severe CNS involvement in SLE is uncommon with varying manifestations including acute confusion, psychosis, seizures, aseptic meningitis, transverse myelitis, cranial and peripheral neuropathy. Cerebrovascular insult (CVI) is associated with antiphospholipid antibodies and can be caused by atherosclerosis, hypertension and, in rare cases vasculitis[157]. Neuropsychiatric symptoms in SLE can be due to active SLE disease or secondary to other causes including infections, metabolic abnormalities or medication.

Investigations include cerebral and/ or spinal MRI together with cereospinal fluid (CSF) analysis[158,159]. Electroneurography (ENG) is used for peripheral nerve investigation.

Neuropsychiatric SLE (NPSLE) is associated with poor prognosis and mortality[7,160] and may lead to working disability and impaired quality of life.

Antiphosholipid syndrome (APS)

Arterial and venous thrombosis, thrombocytopenia and pregnancy complications, such as recurrent fetal loss, together with persistent presence of anti-cardiolipin antibodies and/or positive lupus anticoagulant (LAC) test, constitute the condition APS[161]. APS occurs alone (primary APS) or together with autoimmune disorders such as SLE.

Anti-cardiolipin (aCL) antibodies of any kind (aCL-anti-β2-glycoprotein I-antibodies or LAC) occur in approximately 15-40% of SLE patients, depending on method and definition[162]. About 1/3 of SLE patients develop clinical APS[163]. As mentioned in the sections above, aCL antibodies are associated with Libmann-

Sacks endocarditis and CVI, but also with SLE-related migraine acute myocardial infarction and pulmonary hypertension[161].

Diagnosis and classification

SLE diagnosis is based on organ manifestations characteristic of SLE together with typical immunopathology. Diagnostic principles were suggested in 1975[164] which include presence of a multisystem disease, in at least two organ systems together with a positive ANA test. These principles have been modified to include the absence of other conditions that could better explain the symptoms, which improves diagnostic specificity and sensitivity[7,8]. The American College of Rheumatology (ACR) classification criteria for SLE from 1982[49] are widely used in clinical studies which facilitates study comparability. In the majority of studies of SLE, patients fulfilling at least 4 ACR classification criteria are included. The 1982 ACR classification criteria were updated in 1997[165] when LE cells were excluded and lupus anticoagulant test as well as anti-cardiolipin antibodies were included. The 1997 ACR criteria were validated in 2012[54]. Over the years, new knowledge in the field of SLE and immunology has been added and an overhaul of the classification criteria was needed to ensure clinical relevance. Due to this, new classification criteria for SLE were suggested in 2012 by the Systemic Lupus International Collaborating Clinics (SLICC) group[54] requiring a) at least four criteria fulfilled including one clinical- and one immunological criterion or b) SLE specific, biopsy verified glomerulonephritis as the only clinical criterion together with positive ANA or presence of anti-dsDNA antibodies. The new classification criteria include low complement component levels, commonly seen in active SLE and myelitis as well as acute cutaneous lupus, important SLE manifestations missing in the earlier 1982 and 1997 ACR classification criteria. Autoantibodies have a diagnostic value. The ANA test has a low specificity for SLE, is positive in healthy individuals and in several other conditions including infections and other autoimmune diseases. The ANA test is positive in 97-99% of the SLE patients and presence of ANA is one of the classification criteria for SLE. Anti-dsDNA antibodies have a high specificity for SLE and are also part of the SLE classification criteria.

Estimation of disease activity

When the SLE patient with certain clinical signs and symptoms presents in clinical practice, the physician first has to decide whether those are due to lupus disease activity or caused by other conditions such as infection, drug side effects, depression or fibromyalgia. Some of these conditions are common comorbidities in SLE. If assessed to derive from SLE disease activity, distribution and severity of disease has to be determined in order to choose adequate therapy. Correct and comparable measures of disease activity are not only important in the clinical setting but also in research. Because of the heterogeneity of SLE manifestations,

standardized indices have been developed. Systemic Lupus Erythematosus Disease Activity Index (SLEDAI)[166] and European Consensus Lupus Activity Measure (ECLAM)[167] have been validated for retrospective use[168,169]. The SLEDAI 2000 (SLEDAI-2K)[170] is a modification of SLEDAI, on which it is extensively based and is also a measure of persistently active disease. It is a global index with clinical and laboratory weighted items, often used in clinical studies. Other validated indices of SLE disease activity are the British Isles Lupus Assessment Group Index (BILAG)[171] and the Systemic Lupus Erythematosus Activity Measure (SLAM)[172].

SLE treatment

Treatment in SLE is based on the type of organ involvement and severity of disease exacerbation. The goal of treatment is to induce remission, and therefore reduce disease flares and minimize corticosteroid treatment to avoid future organ damage, improve long-time survival and quality of life. Furthermore, side-effects to therapy should be prevented. If flares are minimized, comorbidity is reduced.

Severe inner organ involvement is treated with potent immunosuppressive therapy as the initial treatment, followed by remission sustaining therapy.

Non-steroidal anti-inflammatory drugs (NSAID)

Mild flares where the most prominent symptoms are arthralgia and myalgia, as well as general symptoms such as fever and malaise can be treated with NSAIDs for shorter periods. However, the risk for CVD and renal damage with long term treatment must be considered[173,174].

Antimalarial drugs (Hydroxychloroquine and Chloroquine phosphate)

Antimalarial drugs reduces photosensitivity and skin manifestations, as well as musculoskeletal symptoms, including arthritis. Furthermore, they have been used to achieve sustained remission and improve long-term survival[175]. Moreover, hydroxychloroquine (HCQ) improves lipid profile[176]. Retinopathy is an unusual side effect of antimalarials, more often reported in Chloroquine phosphate and therefore, HCQ is preferred[177].

HCQ reduces acidity in lysosomes, affecting TLR 7 and TLR 9 mediated inflammation in APCs, resulting in inhibition of IFN α production that is initiated by DNA or RNA containing ICs in pDCs. Furthermore, HCQ is described to hamper platelet activation and aggregation[178]. Aspirin and HCQ administered together have synergistic effects on inhibition of platelet activation[179]. Moreover, the anti-thrombotic effects of HCQ in APS is well known[180].

Corticosteroids

Corticosteroids in SLE are used for rapid reduction of inflammation and are recommended for use together with other treatment with slower onset.

Limited cutaneous manifestations can be treated with local steroids. Milder flares with skin and musculoskeletal symptoms as well as serositis are treated with low to moderate doses of corticosteroids per os.

High dose corticosteroid treatment is used during induction treatment of severe inner organ involvement including kidney and CNS.

Calcium and vitamin D therapy, and in certain cases bisphosphonates, should be initiated together with steroids to avoid osteoporosis. Monitoring of other side effects of steroid treatment such as hyperlipidemia, elevated blood pressure, diabetes, cataract, glaucoma and infections are also important[181,182].

Intravenous immunoglobulin treatment (IvIg)

Serious forms of antiphospholipid syndrome (APS) can be treated with IvIg. The treatment is also indicated in autoimmune hemolytic anemia and severe thrombocytopenia[183,184].

Immunosuppressive drugs

Azatioprin (Aza) is used as corticosteroid-sparing therapy in various SLE manifestations including skin, musculoskeletal symptoms and serositis. It is also used as maintenance therapy in glomerulonephritis[185]. Aza is a purine analogue and its metabolites inhibit DNA synthesis, leading to impaired proliferation of immune cells[186].

Arthritis in SLE is treated with Methotrexate (Mtx). Mtx is also used as steroid-sparing therapy in other manifestations such as skin rash and serositis, but has no place in induction therapy for severe inner organ disease[187]. Mtx is a folic acid analogue that exerts its effects through competitive inhibition of dihydrofolate and inhibition of RNA and DNA synthesis.

Mykofenolat mofetil (MMF) is a first line induction treatment in proliferative and membranous lupus nephritis[188] and can be used as maintenance therapy instead of azathioprine after induction treatment with MMF or cyclophosphamide (CTX)[185]. In women of childbearing age, MMF is chosen before CTX in cases where fertility should be preserved. The active metabolite in MMF blocks proliferation of activated B- and T cells[186].

Cyclophosphamide (CTX) is used as induction treatment of severe inner organ involvement. It is the first line treatment in SLE glomerulonephritis ISN class III-IV A and A/C with or without class V. In accordance with the Euro Lupus protocol, lower doses of intravenous (iv) CTX together with corticosteroids are preferred in order to reduce the cytotoxic side effects[189]. For SLE patients with severe CNS involvement, higher doses of iv CTX are needed[190]. CTX is an alkylating cytotoxic drug with severe side effects including infections, infertility, hemorrhagic cystitis and malignancies. It exerts its effect through affecting DNA and RNA, resulting in reduced cell division.

Therapies that target immune cells

Belimumab is a monoclonal antibody against B lymphocyte stimulator (BLyS), that binds to BLyS and inhibits its receptor-mediated actions. For SLE patients with non-renal, moderate- to high disease activity, who are not responding to conventional therapies, Belimumab is an adjuvant treatment of choice. The therapy is preferred in patients with oral or nasal ulcers, skin-or musculoskeletal manifestations and in certain cases hematological disorder, together with serological activity, *e.g*. positive for anti-dsDNA antibodies and low complement.

Rituximab (RTX) is a recommended treatment for SLE glomerulonephritis in international society of nephrology (ISN) class III-IV A or A/C, with or without presence of class V nephritis, after treatment failure with CTX or MMF[191]. However, there is no evidence of efficacy of this treatment from randomized controlled trials.

RTX is a monoclonal antibody against CD20, an antigen specific for B cells. Since CD20 is not expressed on plasmacells, these are not depleted.

Anti-IFN α receptor antibodies and antibodies against type I IFNs

Therapies directed against type I IFNs or the interferon alpha receptor (IFNAR) are discussed in the section about interferons.

To summarize, SLE patients should be closely monitored for early detection of disease flares to enable adequate treatment and achieve remission. If tolerated, patients should receive long-term treatment with antimalarilals, that has been shown to prevent disease exacerbation and reduce mortality[192]. Long-term corticosteroid treatment should be avoided but when necessary, as low dose as possible should be used. In the future, new therapies to replace corticosteroids and immunosuppressive treatment are warranted.

Estimation of organ damage

Being a chronic, multi-organ disease with recurrent flares, cumulative disease activity over time, as well as medication, can lead to irreversible organ damage. In order to avoid permanent organ damage and organ dysfunction, close monitoring of SLE disease, with early detection and adequate treatment of disease exacerbation is most important. In SLE, organ damage is determined by the Systemic Lupus International Collaborating Clinics (SLICC)/ACR Damage Index (SDI)[193] an index that has been thoroughly validated[194,195]. The damage index scores irreversible organ damage that occured after SLE diagnosis, regardless of cause. In order not to score ongoing inflammation as damage, the organ damage must have been persistent for at least 6 months.

Disease course and prognosis

Survival in SLE has improved markedly during the last decades but a more than 3 fold increased estimated mortality risk is still seen compared to the background population [196]. With prolonged survival of the patients, pattern of diseases has changed over the years with increased cardiovascular morbidity[196]. High SDI score predicts mortality[197-199] and it is therefore important to understand how SDI occurs in order to prevent it. Pre-existing organ damage, older age, African ethnicity, steroid use and hypertension are associated with development of new organ damage[199,200]. Other unfavorable prognostic factors are male sex, kidney and CNS disease involvement[199,201]. Treatment with antimalarials protect from organ damage, including coronary heart disease and mortality[196,199,200].

In a recent, large prospective study, 40% of the patients had developed organ damage one year after diagnosis and over 69% of the patients had developed organ damage after a mean disease duration of 16 years. A progressive linear increase of organ damage was seen over the years, confirming observations from other studies[200,202,203]. Antiphospholipid syndrome and presence of anticardiolipin antibodies predicted onset of neuropsychiatric damage, consistent with previous findings[204]. The organ systems most frequently damaged were eyes, musculoskeletal and neuropsychiatric[202]. Similar results have been found by others[204]. Cardiovascular disease occurs later in disease course with 10% of the patients affected after five years and 20% after thirty-five years of follow-up in a long time follow up study of SLE patients with a mean age at diagnosis of 33 years[202]. Besides irreversible organ damage, disability, including loss of working ability and impaired quality of life are long term outcomes in SLE studies. Health related quality of life (HRQOL) can be measure by The Medical Outcomes Survey 36 (SF-36), a validated questionnaire on self-perceived health that has been used in SLE outcome studies[205-207].

Cardiovascular disease in SLE

Overview

Cardiovascular disease is a wide group of diseases of the heart and vascular system of which ischemic stroke (ischemic cerebrovascular disease, ICVD), coronary heart disease (CHD) and peripheral artery disease are investigated in relation to SLE in this thesis[208]. In the western world, atherosclerosis and its complications are the leading cause of death[209,210]. The majority of clinical cardiovascular events are due to atherosclerosis, a condition developed through an interplay between risk factors such as hypertension, smoking and dyslipidemia, and the immune system, where both innate and adaptive immunity play roles. A progressive increase in cardiovascular disease (CVD) risk is seen with older age[211].

Endothelial dysfunction and atherosclerosis

The artey wall is composed of three layers; the inner layer, the intima, the media and the adventitia. The intima is composed of the inner endothelium and underneath, a thin, fibrocollagenous structure. The media consists of smooth muscle cells and elastic fibers and the adventitia comprise mainly collagen.

The endothelium and endothelial function.

Endothelial dysfunction (ED), is a condition characterized by decreased synthesis, release and activity of endothelial derived nitric oxide (NO), impaired endothelial dependent vasodilatation and increased coagulation[212,213]. ED is considered an initial, reversible step in atherosclerosis development, indicating atherosclerotic risk[213]. Endothelial cell activation is a pro-inflammatory state with elevated expression of cell surface adhesion molecules, closely linked to ED[214]. The endothelium is the inner cell layer of the heart and vasculature that has regulatory effects on coagulation, vascular tone and angiogenesis and plays an essential role for vascular homeostasis[214]. During normal conditions, the endothelium prevents endothelial-leukocyte- and platelet-adhesion to the vascular wall and endothelial cell-derived nitric oxide (NO) is an important mediator in this process. By inhibition of the transcription factor NF-κB, NO contribute to decreased expression in ECs of cell adhesion molecules, including intercellular adhesion molecule-1 (ICAM-1), vascular cell adhesion molecule-1 (VCAM-1) and endothelial leukocyte adhesion molecule (ELAM, also called E-selectin)[212]. Activated ECs, on the other hand have an increased expression of these adhesion molecules. Besides leukocyte adhesion, with T cells and monocytes sticking to the

vascular wall, activated endothelium promote platelet aggregation, vasoconstriction and smooth muscle proliferation[214]. Endothelial cells (ECs) can be activated by pro-inflammatory cytokines including TNF α and interleukin-6[214,215]. Not only cytokines, but also high cholesterol blood levels can lead to endothelial cell activation, first demonstrated in animal models[216]. Hypertension, hypercholesterolemia, oxidative stress or external factors such as smoking, can contribute to ED[215]. These factors have also been shown to lead to endothelial cell activation[217]. Turbulent blood flow, often seen during physiological conditions in areas of vascular branching, can cause endothelial cell activation because of lowered levels of endothelium-derived NO[214]. Atherosclerotic lesions are more often seen at sites of vascular branching compared to vascular areas with laminar blood flow[214].

Atherosclerosis pathogenesis

Atherosclerosis is a systemic, chronic, slowly progressing inflammatory disease initiated by adhesion of leukocytes to the activated endothelium. Increased permeability of the activated endothelium, facilitates migration of leukocytes, monocytes being most abundant, through the endothelium into the intima, where they differentiate and mature[215]. LDL molecules penetrate the intima and are bound there by apolipoprotein B100- interaction with proteoglycans of the extracellular matrix. LDL is then modulated by enzymes to oxidized LDL (oxLDL) that contribute to further activation of ECs and macrophages. Monocytes, developed into macrophages in the intima, upregulate scavenger receptors, engulf oxLDL and turn into foam cells[218]. An asymptomatic, fatty streak is formed, that, depending on circumstances, can either disappear, or progress into an atheroma e.g an atherosclerotic lesion, consisting of a local thickening of the intima comprising immune cells, lipids and connective tissue[210]. Cells of the innate immune system; monocytes, mast cells and dendritic cells (DCs), that migrate to the intima, attracted by chemokines and cell adhesion molecules on ECs, express pattern recognition receptors (PRR) like TLRs on their surface. The TLRs bind molecules with pathogen-like molecular pattern such as oxLDL, bacterial toxins, and heat shock proteins (HSPs). The TLR mediated molecular binding on immune cells initiate cell activation and production of proinflammatory cytokines, such as IL-1β and TNF-α[210], contributing to a more inflammatory environment in the artery wall. Antigens, including oxLDL, presented by DCs and macrophages are recognized by specific T cells in the atheroma, triggering an adaptive immune response. Activated T cells produce cytokines, such as IFN-γ, leading to further macrophage and EC activation[218]. Production of extracellular connective tissue is enhanced and smooth muscle cells (SMCs) from the media migrate into the intima. Outside the necrotic, lipid rich core of the atherosclerotic lesion, a fibrous cap is built[215] and a stable plaque is formed.

Activated immune cells in the atheroma, T cells, macrophages and mast cells, produce TNF-α and IFN-γ, that degrade the stable fibrous cap that turns into a site vulnerable for rupture; a vulnerable plaque. Metalloproteinases, produced by activated macrophages and mast cells, also contribute to this degradation. A vulnerable plaque is more prone to rupture, leading to exposure of prothrombotic substances in the subendothelial space to the blood flow. In this way, subendothelial, prothrombotic tissue factor is exposed to the circulating blood with subsequent platelet aggregation, activation of the coagulation cascade and thrombus formation on the ruptured plaque[210].

Epidemiology

Incidence of cardiovascular disease (CVD) is declining in the general population, after adjustment for age, due to decreased smoking and improved primary prevention including treatment of hypertension and dyslipidemia[219,220].

A bimodal pattern of mortality in SLE was described in the mid-1970s with early mortality caused by active SLE disease and infections and later deaths due to myocardial infarction[221]. Thirty years later, mortality in active SLE has declined due to efficient immunosuppressive therapy and better treatment of comorbidities, however, deaths caused by CVD and infection are still the main causes of death in SLE[222]. Patients with SLE have a 2 to 10-fold elevated risk of acute myocardial infarction (AMI), as compared to the background population[223,224]. Observation of standardized mortality rate caused by CVD in SLE patients vary between 1.7 and 3.0[225,226]. In a large epidemiologic study, women with SLE aged 35-44 years had a 50-fold increased relative risk of AMI, compared to age-matched women in the Framingham Offspring Study[227]. Results from a population based Swedish study demonstrated a 16-fold increased risk of death from coronary heart disease in SLE patients 20-39 years of age compared to age-matched controls in the general population[226]. Furthermore, in a large, longitudinal study of SLE patients, the excess risk of cardiovascular events (CVE) was higher in the patients of the cohort younger than 40 years, consistent with findings in other studies[227-229]. However, the absolute risk of AMI in the younger age groups was, low[227].

Traditional risk factors

Risk factors for CVD are well characterized and include smoking, hypertension, dyslipidemia, diabetes, age, male sex, family history of premature CVD and genetics. Furthermore, chronic kidney disease, low education and socioeconomic status contribute to elevated CVD risk [208]. Lipid dysregulation with high total cholesterol (TC), elevated low density lipoprotein (LDL) and triglycerides (TGs)

and low high density lipoprotein (HDL) levels increase the CVD risk with LDL lowering as the primary target of lipid lowering treatment[230]. Besides their LDL-lowering effect, statins are observed to have anti-inflammatory properties[231].

Traditional cardiovascular risk factors in SLE

The prevalence of traditional cardiovascular risk factors is higher in SLE patients compared to healthy controls[232-234]. The metabolic syndrome, a condition with abnormal glucose metabolism, central obesity, lipid dysregulation and hypertension, is a predictor of cardiovascular morbidity and mortality[235] and there is an increased prevalence in SLE patients compared to healthy controls[234]. Sedentary lifestyle, early menopause, hypertension[236-238] and diabetes mellitus as well as increased levels of VLDL and TG have been observed in SLE[232]. Smoking is a strong predictor of CVD[239,240] and is a risk factor for progression of coronary artery calcification in SLE[241]. Increased homocysteine levels are considered a CVD risk factor in the general population[242]. In SLE patients, high homocysteine levels are associated with atherosclerosis, coronary artery disease and thrombotic events[243,244] as well as with subclinical atherosclerosis[233,245]. In a recent meta-analysis, low HDL, elevated TGs and age, significantly affected CIMT, a measure of subclinical atherosclerosis, in SLE patients[246].

Since lipid profile in SLE is investigated in paper IV in this thesis, lipid dysregulation is described in more detail in a section below.

SLE specific risk factors

The increased CVD risk seen in SLE patients remains also after adjusting for traditional CVD risk factors[223]. A possible interaction between traditional- and disease-related risk factors and factors yet to be discovered might promote atherosclerosis development in SLE.

Type I IFNs and endothelial function

Type I IFNs, key cytokines in SLE pathogenesis, are implicated in development of CVD through several mechanisms. First of all, type I IFNs, which induces inflammasome-mediated IL-18 production, inhibits the differentiation and maturation of EPCs into ECs, thus, impairing vascular repair[247]. The observed decreased in the number and function of endothelial progenitor cells (EPCs) together with an impaired ability in EPCs to differentiate and mature into ECs has been observed *in vitro*[248]. The ECs, affected by IFN-α, had an abnormal phenotype and an impaired capacity to produce proangiogenic molecules[248]. Endothelial function was impaired in SLE patients compared to healthy controls[249]. In a study investigating SLE patients, depletion of EPCs in peripheral blood was seen as

compared to healthy controls and the depletion was more pronounced in patients with high type I IFN levels. Furthermore, endothelial dysfunction, measured by Endopat in finger arteries, was associated with elevated type I IFN levels, supporting the findings in experimental mouse models and *in vitro* [250]. In addition, young SLE patients, under the age of 50 years, were demonstrated to have elevated levels of circulating apoptotic ECs in peripheral blood as compared to healthy controls, that correlated with endothelial dysfunction, measured by brachial FMD[251]. In murine models of lupus and atherosclerosis, loss of type I IFN signaling resulted in increased endothelial-dependent vasorelaxation, elevated EPC number, improved EPC function and decreased atherosclerosis[252]. More pronounced coronary artery calcification has been observed in SLE patients with a type I IFN signature[253] supporting the role of type I IFN in atherosclerosis development.

Other mechanisms for type I IFNs to affect the vasculature are described: a recent study indicate that type I IFN might prevent smooth muscle progenitor cells (SMPCs) from differentiating into mature smooth muscle cells (SMCs) with a potential influence on atherosclerotic plaques-vulnerability[254]. Type I IFNs seem to augment inflammation and plaque instability in the atherosclerotic lesion by other mechanisms as well: in the atherosclerotic plaque, pDCs produce type I IFNs. In the presence of bacterial lipopolysaccharide (LPS), type I IFNs have been reported to upregulate synthesis of TNF-α, IL-12 and MMP-9, all involved in plaque degradation[255].

Type I IFNs also affect inflammatory cells with an impact on the endothelium : type I IFNs promote macrophage-endothelial cell adhesion[256] and enhance macrophage lipid uptake and foam cell formation,[257]promoting the atherosclerosis process. Furthermore, a type I IFN signature has been found in platelets from SLE patients. This signature was associated with a history of CVD. Platelets with the type I IFN signature appeared to be more activated and might have a prothrombotic phenotype, which may contribute to CVD[258].

In all, the impaired endothelial function seen in SLE might be due to type I IFN activity. Type I IFNs and their receptors may be therapeutic targets to decrease ED and CVD in SLE in the future and monitoring of type I IFNs may be important in assessing CVD risk in SLE patients.

Inflammation and organ damage

Current disease activity is associated with elevated CVE risk[228,259]. Pro-inflammatory cytokines such as TNF-α and IL-6 are demonstrated to be associated with CVD in SLE, besides the type I IFNs[260,261]. High-sensitive CRP is associated with progression of subclinical atherosclerosis[241].

Not only current disease activity, but also organ damage, is reported to be associated with increased CVD risk[259]. Elevated frequency of atherosclerosis was seen in SLE patients with glomerulonephritis compared to patients without renal involvement and healthy controls[262]. Glomerulonephritis is associated with conditions related to elevated CVD risk including hypertension, dyslipidemia, treatment with high dose steroids, proteinuria and/or nephrotic syndrome leading to a hypercoagulable state. In a recent study, only hypertension was associated with atherosclerosis after adjustment for age and sex[262]. Glomerulonephritis often affects relatively young SLE patients[263], so early appropriate treatment of glomerulonephritis and CVD risk factors including hypertension is crucial to decrease the risk for atherosclerosis development. Impaired renal function, as a possible damage after SLE glomerulonephritis is associated with CVE in SLE, indicating that estimation of glomerular filtration rate (GFR) should be part of the screening for CVD risk factors in SLE patients[264].

NETs are suggested to be involved in atherosclerosis development by affecting the endothelium and the type I IFN system[265,266]. Moreover, NETs can activate platelets and might thereby contribute to coagulation and thrombosis[267,268].

Antiphospholipid antibodies

Antiphospholipid antibodies (aPLs) are autoantibodies directed against complexes of phospholipids and phospholipid-bound proteins[269,270] with different, but to some extent overlapping specificities. In clinical practice they are measured in three ways: anticardiolipin antibodies (aCLs) of IgG or IgM type, autoantibodies to β2-glycoprotein 1 (β2GP1) and lupus anticoaculans (LA). Presence of aPL was related to arterial and venous thrombosis[271-273] and their function is not totally clear. aPLs stimulated platelet activation and aggregation[273-277], affected factors in the coagulation cascade and were involved in monocyte-and endothelial cell activation[278-282]. Further, aPL can promote NETosis in PMNs, a mechanism that might contribute to thrombosis in antiphosholipid syndrome (APS)[283]. Presence of aPL has been found to be associated with atherosclerosis and CVD in SLE[233,269]. In a prospective study of SLE patents, antiphospholipid antibodies (aPL) were independent predictors of CVE[239], in line with previous findings[284].

Medication

Treatment with glucocorticoids in SLE is a double-edged sword in relation to CVD risk. On the one hand, accurate treatment of disease activity and inflammation might protect against atherosclerosis as inflammation and inflammatory markers are associated with increased CVD risk[233,285]. Furthermore, protective effect of steroids in atherosclerosis has been demonstrated in an animal study[286]. On the other hand, steroid treatment increases the risk for central obesity, increase cholesterol levels, insulin resistance, diabetes, osteopenia and

osteoporosis, conditions associated with CVD[287]. Several studies have demonstrated an acute effect of steroids on CVE risk and cumulative use in the past seems to have lower impact on this risk[228,288-290]. A dose-dependent increased risk of CVE was seen in SLE patients on current treatment with glucocorticoids, in a large longitudinal study, also after adjusting for blood pressure, serum cholesterol and current disease activity[228]. A dose of 20 mg/day or more was associated with a 5-fold elevated CVE rate after adjustment for age[228]. The association between high steroid dose and CVE can, to some extent, be due to high SLE disease activity if adjustment for this is not performed. Data are, however contradictive. Other studies have shown that the cumulative steroid dose was important for development of atherosclerosis[291,292] and a low average steroid dose has been observed to be protective for atherosclerosis[259].

Screening for CVD risk factors is essential to reduce CVD risk in the population and in SLE patients. The aims in SLE patients are early detection and treatment of hypertension, hyperlipidemia and diabetes together with reduction of disease flares and inflammation. Patients should be informed about the benefits of lifestyle changes when needed and avoid smoking, sedentary lifestyle and being overweight. Patients should be educated to avoid environmental factors that trigger flares such as ultraviolet light.

The use of antimalarials, e.g. hydroxychloroquine, has been observed to be protective against CVD in SLE, and cholesterol lowering effects are suggested to contribute[293,294]. Moreover, decreased thrombotic risk associated with hydroxychloroquine treatment has been reported[295,296]. However, there are conflicting results: in a large, longitudinal study, a protective effect of treatment with antimalarials was not seen[228].

In all, close control of SLE disease activity and continuous treatment with hydroxychloroquine, if tolerated, together with a steroid dose, at as low as possible for disease control, might prevent CVD in SLE.

Genetics

Certain gene polymorphisms are associated with elevated CVD risk in SLE[297]. An example of this was demonstrated in a Swedish study, where signal transducer and activator of transcription factor 4 (STAT4), a risk allele for SLE, was associated with elevated risk for arterial events[298] and ischemic cerebrovascular disease in SLE patients[299]. In another study investigating single-nucleotide polymorphisms (SNPs) associated with coronary heart disease (CHD), the interferon regulatory factor-8 (IRF8) gene was demonstrated as a susceptibility locus[300].

Lipid dysregulation in SLE

Lipoprotein abnormalities are characteristic of SLE and are seen in newly diagnosed, untreated SLE patients. The lupus pattern comprising elevated levels of triglycerides (TG), very low density lipoproteins (VLDL) and decreased high density lipoproteins (HDL) concentrations, varies with disease activity with a harmful lipid profile in active disease[301]. Hypercholesterolemia was seen in 36% of newly diagnosed SLE patients in the Systemic Lupus International Collaborating Clinics cohort of 918 patients [302]. 278 of the patients were followed for 3 years. At the 3 year follow up, hypercholesterolemia was seen in 60% of the patients[303]. The mechanism behind the abnormal lipoprotein and lipid profile in SLE is multifactorial. Cytokines including TNF α contribute to hypertriglyceridemia[260]. Reduced function of lipoproteinlipase (LPL)[304] due to impaired clearance of chylomicrons, leading to VLDL and chylomicron elevation followed by increased TG and lowered HDL levels,[305]is described. Furthermore, antibodies including anti LPL[306] and antibodies to oxidized LDL (oxLDL) were observed[307]. Elevated levels of pro- atherogenic oxLDL and pro-inflammatory HDL (piHDL) were reported to be associated with the abnormal lipoprotein pattern in SLE[308,309].

Apolipoprotein M

Human apolipoprotein M (apoM) is a 25 kDa molecule and part of the lipocalin protein superfamily that was first described in 1999[310]. It is mainly expressed in liver and kidney and the greatest part is bound to HDL in plasma, but a minor part is bound to other lipoproteins such as LDL and VLDL[310]. ApoM is present in approximately 5% of HDL particles[310]. The structure consists of an 8-stranded antiparallel β-barrel containing a hydrophobic binding pocket where sphingosine-1 phosphate (S1p) is bound[311]. ApoM is the physiological carrier of S1P, a circulating lipid mediator with various biological functions including modulating effects in the immune and vascular system[312]. In plasma, the majority (65%) of S1P is bound to apoM with the remaining 35% bound to albumin[313]. S1P signals through 5 G protein-coupled receptors S1P1-5 and the receptors have different roles depending on cellular expression. The S1P1 receptor is expressed on endothelial cells and is needed for maintaining endothelial barrier function as well as for regulation of lymphocyte migration[312]. Increased permeability of the endothelium is seen during infection and inflammatory conditions and is characteristic of endothelial dysfunction[314]. Disrupted endothelial barrier function admits passage of lipids to the subendothelium together with migration of monocytes that differentiate to macrophages with subsequent macrophage uptake of lipids and formation of foam cells and lipid deposition in the subendothelial space and development of manifest atherosclerosis and plaque formation[314]. ApoM carries S1P to its receptor on endothelial cells promoting endothelial barrier

function, being a vasculoprotective component of HDL[313]. By binding oxidized phospholipids, apoM-containing HDL has increased anti-oxidant properties compared to HDL without apoM. Furthermore, apoM containing HDL enhance cholesterol efflux from peripheral tissue more efficiently than HDL with no apoM content[315,316]. The role of apoM-S1P complex in atherosclerosis has been demonstrated in vivo, where mice with no aortic S1P1 receptors developed atherosclerotic plaque and more macrophage infiltration in the vessel wall compared to mice with functioning S1P1 receptor. It was also demonstrated that human umbilical vein endothelial cells (HUVECs) exposed to HDL containing apoM bound to S1P had decreased ability to express intracellular adhesion molecule-1 (ICAM-1) in respons to TNF α exposure, showing an anti inflammatory role on the endothelial cells for apoM-S1P[317]. Similar findings has been shown in human aortic endothelial cells (HAEC) treated with TNFα where E-selectin and vascular cell adhesion molecule-1 (VCAM-1) reduction was caused by apoM bound S1P[318]. ApoM has been shown to be necessary for formation of preβ-HDL, a subclass of lipid- poor apolipoprotein that contribute to transport of cholesterol from tissue to liver. Overexpression of apoM in LDL receptor knock out mice exposed to cholesterol rich diet protected from atherosclerosis[319].

To summarize, apoM is the carrier of S1P, a molecule with modulatory effect on the endothelium, indicating a role for apoM in vascular homeostasis.

Assessment of subclinical atherosclerosis

Detection of subclinical atherosclerosis is crucial to prevent cardiovascular events (CVE). If subclinical atherosclerosis is detected before onset of clinical manifestations e.g. myocardial infarction or stroke, intense primary preventive treatment can be initiated in order to prevent cardiovascular events. Assessment of subclinical atherosclerosis is therefore important in SLE patients to prevent CVE. Subclinical atherosclerosis can be detected by measurement of carotid intima media thickness (CIMT) or by CT scanning of the chest evaluating coronary artery calcification (CAC) or by brachial flow mediated dilatation (FMD), measuring peripheral artery reactivity by a non-invasive method[208]. Peripheral arterial tonometry (PAT), can be measured by a device named Endopat, and is another non-invasive method to assess endothelial dysfunction (ED), that is operator-independent[320-322]. In a recent meta-analysis evaluating non-invasive measurement of ED in relation to CVD, both pathological values of brachial FMD and Endopat, were observed to be independent predictors of CVD and all-cause mortality[323].

The platelets

Background

Platelets are small anucleated cell fragments, derived from megakaryocytes in bone marrow or, as recently described, lung[324]. With approximately 140-400 x 10^9 cells/L blood, platelets are the second most common blood cell, only defeated by the erythrocyte, and have an approximate life span of 5-7 days. Platelets have important functions in vascular hemostasis, thrombosis formation and inflammation[325-328]. Despite the lack of a nucleus, platelets contain megakaryocyte-derived mRNA and are able to undergo protein synthesis[329,330]. Platelets contain three different kinds of granules: α-granules, dense granules and lysosomal granules containing activating molecules and cytokines. Platelets exert their effects through these granules, through cell-cell interaction and platelet-derived microparticles (PMP) that will be described below[331].

The platelet in vascular homeostasis, thrombosis and cardiovascular disease

Exposure of subendothelial collagen after vascular injury such as atherosclerotic plaque rupture, leads to a rapid response from circulating platelets. Platelet glycoproteins interact with collagen and collagen-bound von Willebrand factor, leading to change of platelet shape, as well as tight adhesion of platelets to the damaged vessel wall and subsequent platelet activation. The activated platelets will aggregate and form a thrombus with a fibrin-containing core. The activated platelets can recruit new, circulating platelets by secretion of aggregation-stimulating mediators and these platelets adhere to the growing thrombus, and an unstable shell, surrounding the core is formed [332,333]. Platelet aggregation and thrombus formation can lead to total arterial occlusion with subsequent myocardial infarction or ischemic stroke[334]. Different mechanism are proposed to contribute to increased thrombus formation in SLE patients. For example, platelets can be activated through IC-FcγRIIA interactions leading to a pro-thrombotic state with increased thrombus formation[335]. Moreover, a potential connection between the lectin complement pathway, platelets and the coagulation system has been demonstrated in a recent study[336] and suggested to play a role in thrombus formation in SLE patients. The proteases MASP-1/2 of the lectin complement pathway are known to activate coagulation factors, but the initiators of the MASP-1/2 activation has been unknown. In this study, activated platelets and fibrin were demonstrated to initiate activation of MASP-1/2 during thrombosis formation *in vitro* and *in vivo*. A correlation between activated MASP proteins, activated platelets and the contact coagulation pathway was seen in SLE patients, most pronounced in those with a history of thrombosis.

Another mechanism that may contribute to the elevated CVD risk seen in SLE patients is an upregulation of type I interferon-regulated genes, demonstrated in

platelets in SLE patients that was most pronounced in patients with CVD comorbidity[258].

The platelet in inflammation

Platelets have the capacity to modulate immune response by their surface receptors. TLRs are expressed on the platelet surface and when recognizing a pathogen, the platelets are activated, form aggregates and may trap the pathogen. Furthermore, platelet granules, comprising immune-stimulating and microbicidal substances, are released during platelet activation to combat the pathogen. In rheumatoid arthritis (RA), platelet-derived microparticles containing IL-1 are found in synovial fluid[337]. Platelet-derived microparticles (PMPs) are small membrane-enclosed particles formed upon cytoplasmic membrane blebbing, enabling platelets to act at inflammatory sites distant from the cell itself[338], containing ligands, receptors, enzymes and cytokines from the mother cell. The microparticles in the arthritic joint can induce production of inflammatory molecules in fibroblasts[339]. PMPs from activated platelets are actors in the pathogenesis of other autoimmune and inflammatory conditions. During infection they act in host defense by recruiting leukocytes to inflammatory sites[340] and inhibit T cell differentiation to IL-17 and TNF γ producing T cells[341]. During the inflammatory process in atherosclerosis, monocytes migrate to the subendothelial space[215] and PMPs are suggested to affect monocyte-endothelial interaction[342].

Platelets interact with several immune cells including monocytes and PMNs[343,344]. Platelet-monocyte aggregates are indicators of platelet activation and platelet-monocyte interactions lead to monocyte production and release of pro-inflammatory cytokines[343]. Platelets-PMN interactions promote NET formation[344]. NETosis lead to exposure of autoantigens such as DNA and histones and induction of type I IFNs.

Platelets also interact with endothelial cells and are suggested to play a role in sustaining endothelial barrier function[345]. Increased vascular permeability due to impaired endothelial barrier function is a prominent feature in infection and inflammation and thrombocytopenia, seen in sepsis and severe infection, might be involved in this process[346]. In a recent study, endothelial cells were activated by platelets from SLE patients *in vitro,* through an IL-1β-dependent pathway[347] and this might be involved in cardiovascular disease development. During platelet-endothelial cell interaction, ICAM-1 is up-regulated in endothelial cells , as well as the NF kappa B pathway and expression of IL-8.

Another way for platelets to affect endothelial cell function is through transfer of genetic material. Platelets can bring microRNA (miRNA) through PMPs into the endothelial cell´s intracellular compartment thus affecting the endothelial cell gene expression and may in this way further contribute to endothelial dysfunction[348].

The platelet in the SLE pathogenesis

Decreased serum and platelet levels of pro-inflammatory serotonin has been observed in SLE and associated with severe disease phenotype including glomerulonephritis (GN) and presence of anti-dsDNA antibodies[349]. Platelets are suggested to release serotonin when activated and low platelet serotonin levels correlated with platelet activation. The pro-inflammatory serotonin might influence the adaptive immune system through promotion of T cell proliferation and activation [350] and may contribute to severe inner organ involvement such as GN.

Increased platelet activation in SLE has been observed in several studies with elevated platelet-leukocyte aggregates, increased P-selectin expression and elevated platelet complement C4d deposition[351-354]. Increased platelet complement C4d deposition is also seen in patients with APS and is associated with arterial and venous thrombosis. Platelets with complement C4d deposition correlate with GN in SLE, indicating a role of activated platelets in renal involvement. Mesangial cells are activated by platelets with induction of cell proliferation and production of TGF-β through CD40-CD154 interactions [355] and TGF-β is involved in the GN disease mechanism.

In SLE, immune complexes (IC) can activate platelets through platelet Fc receptors, as mentioned above, leading to expression of CD40 Ligand (CD40L). CD40L is a transmembrane protein first observed on activated T cells, with the capacity to stimulate B cells and dendritic cells[356]. Its soluble form sDC40L is increased in serum from SLE patients and associated with SLE disease activity[357]. CD40L interacts with CD40 on plasmacytoid dendritic cells (pDCs) and stimulate IFN α production, an important cytokine in the SLE pathogenesis[144]. Further, CD40L derived from platelets promote maturation of myeloid dendritic cells (mDCs), uptake of antigens and activation of CD4+ T lymphocytes[358]. Survival of mDCs are prolonged by the influence of CD40L together with a continued antigen presentation with subsequent T cell activation. In these different ways, platelets may modulate SLE disease course, playing an important role in autoimmunity.

The proinflammatory molecules S100A8/A9 and S100A12

Background

The S100 protein family consists of more than 20 calcium binding proteins of low molecular weight (9-14 kDa)[359]. A subgroup of this family: S100A8, S100A9 and S100A12 are also referred to as calgranulins, or myeloid-related proteins, because of their high expression in cells of myeloid origin[360,361]. S100A8 and S100A9 constitute 40 % of the cytosolic protein content in neutrophil granulocytes and 1% of the protein content in the cytosole of monocytes. S100A12 is also highly expressed in granulocytes with approximately 5% of the cytosolic content being S100A12[362-364]. Mice lacking S100A8 die in utero but S100A9 knock-out mice live and are fertile, indicating an important function for S100A8 in embryogenesis[365,366]. Besides polymorhonuclear cells (PMNs) and monocytes, other cell types such as dendritic cells (DCs) and endothelial cells express S100A8/A9 and S100A12[364,367,368]. Furthermore, S100A8/A9 expression in platelets and macrophages has been described[258,369-371]. S100A8 and S100A9 preferably form stable heterodimers, the S100A8/A9 complex, also referred to as calprotectin or MRP 8/14[372,373]. S100A12 forms homodimers and, at low calcium concentrations, hexamers[374,375]. In the intracellular space, S100A8/A9 and S100A12 are important for cell homeostasis and affect the cytoskeleton through microtubule interaction in a calcium dependent manner[376,377]. The S100 proteins are released from PMNs passively, during cell necrosis or NETosis[149,378] or actively secreted by an alternative non-Golgi route[379] at inflammatory sites.

S100A8/A9 and S100 A12 and rheumatic diseases

Increased serum levels of calgranulins are frequently seen in inflammatory conditions including rheumatic diseases. In rheumatoid arthritis (RA) plasma levels correlate with disease activity[380-383]. Furthermore, S100A8/A9 levels are increased in macrophages in synovial tissue in RA[384]. S100A8/A9 levels correlate to radiographic erosions and have been demonstrated to predict radiographic progression in a longitudinal study on RA patients[382,385] . Once released to the extracellular space, S100A8/A9 complex and S100A12 act as DAMPs in autoimmune disease such as RA[386]. Mechanistically, S100A8/A9 and S100A12 act through TLR-4 and RAGE on granulocytes, monocytes and endothelial cells to enhance production of cytokines important in the RA pathogenesis such as IL-1β, IL-6 and TNFα[80]. The role of S100A8/A9 in the RA pathogenesis has been

demonstrated *in vivo*, where S100A8 and S100A9 were up-regulated in experimental antigen-induced arthritis in mice[387]. Furthermore, S100A9 knock out mice, deficient in S100A8/A9 complex, had decreased joint swelling and less leukocyte infiltration in joints[387].

Serum or plasma levels of S100A8/A9 and S100A12 have also been demonstrated in psoriatic arthritis, systemic juvenile idiopathic arthritis (SJIA) and SLE[367,381,388-390]. In SLE, serum levels correlate to disease activity[96,391].

Measurement of S100A8/A9 (calprotectin) in feces is a validated screening tool for patients with suspected inflammatory bowel disease (IBD) and is also used for non-invasive monitoring of established IBD[392]. Furthermore, fecal-calprotectin has been demonstrated as a biomarker for gastrointestinal (GI) organ involvement in systemic sclerosis[393], that might reduce the need for invasive GI investigations in these patients.

S100A8/A9 and S100A12 and cardiovascular disease

The calgranulins are biomarkers of atherosclerosis and cardiovascular disease. S100A8/A9 predicted development of cardiovascular disease in otherwise healthy women[369] and plasma S100A8/A9 levels correlated to severity of coronary heart disease in patients with type I and type II diabetes mellitus[394,395]. Elevated serum S100A12 levels indicate more severe coronary atherosclerosis in patients with coronary artery disease (CAD), diabetes mellitus and chronic kidney disease [396-398]. Expression of S100A8/A9 in human and murine platelets has been described recently[370]. Data from our group confirm these findings in humans. In SLE patients with previous CVD, platelet levels of S100A8/A9 were increased compared to SLE disease controls and healthy individuals, pointing out platelet S100 proteins as potential markers of CVD in SLE[399]. Furthermore, elevated serum levels of S100A8/A9 and S100A12 have been observed in SLE patients with a history of cardiovascular disease,[97] further described in this thesis (Paper I). Not only are the calgranulins valuable biomarkers of CVD, but they are also suggested to be actively involved in atherosclerosis and cardiovascular disease pathogenesis. RAGE plays a role in vascular inflammation and atherosclerosis[400]. The S100A8/A9 complex binds to human microvascular endothelial cells (HMEC-1) and activate a thrombogenic response with elevated transcription of cytokines such as IL-8 and adhesion molecules, e.g. intercellular adhesion molecule 1 (ICAM-1)[401]. Through activation of TLR4 and RAGE on endothelial cells, S100A8/A9 contribute to disturbed endothelial barrier function[81,402]. Besides a potential modulation of endothelial barrier function, S100A8/A9 complex plays an important role in regulation of vascular inflammation and response to vascular damage by various mechanisms. Three experimental disease models were

performed in S100A9 deficient mice lacking S100A8/A9 complex; vasculitis, arterial injury and atherosclerosis. In all these models, impaired recruitment of neutrophils and macrophages to the inflamed area was seen[403], pointing out a role for S100A8/A9 in recruiting inflammatory cells to the vascular wall during different kinds of vascular injury. Other studies indicate that S100A8/A9 induce myeloid cell migration over the vessel wall and increased leukocyte adhesion[404,405]. Furthermore, S100A8/A9 deficient mice have shown reduced production of cytokines, including TNFα and Il-1β and decreased smooth muscle cell proliferation[403], indicating a role for S100A8/A9 in vascular inflammation and in smooth muscle cell infiltration of the intima, during atherosclerosis development. Thus, S100A8/A9 is suggested to mediate an immune response after vascular damage and may in this way contribute to atherosclerosis development (Figure 3).

To conclude, the pro-inflammatory S100 proteins are part of the innate immune system and play a role in both autoimmune-and cardiovascular disease mechanisms. Although innate immunity, including S100 proteins, are essential in our host defense towards pathogens, exaggerated or abberant activation of our immune system could result in unwarranted inflammation and organ damage, including cardiovascular disease and autoimmune disorders.

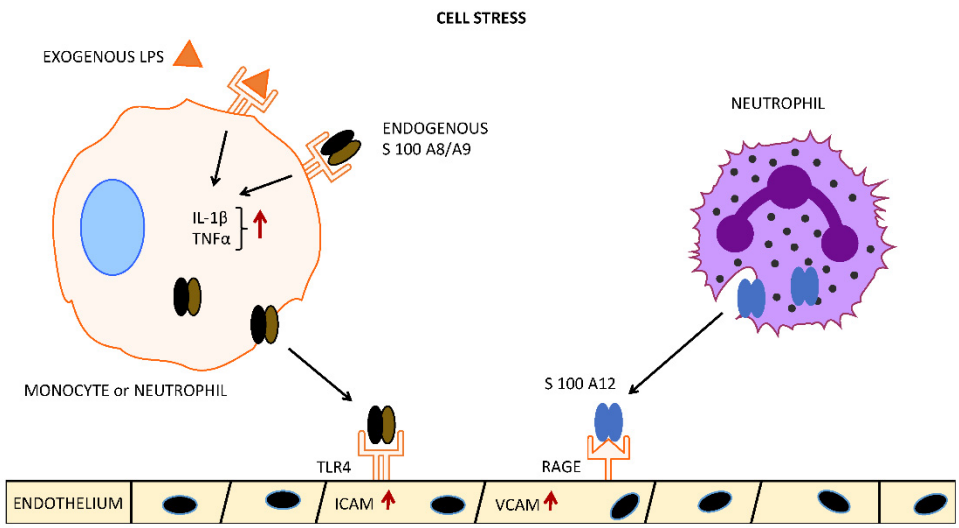


Figure 3 S100A8/A9 and S100A12 mediate inflammation
Lipopolysaccharide (LPS) or S100A8/A9 activate neutrophils through TLR 4 receptors. S100A8/A9 and S100A12, released from activated neutrophils can mediated endothelial inflammation through their respective receptors. *Partially based on biodraw pictures.*

Aims of the present investigation

The overarching aim of the thesis was to study the connections between immunopathology in SLE and cardiovascular disease.

A.) Activation of type I IFNs, key cytokines in the SLE pathogenesis are suggested to contribute to endothelial dysfunction in SLE. Platelets are inflammatory cells and interact with both immune cells and endothelial cells. Since we and others have found increased platelet activation in SLE patients, we wanted to investigate:

1. If SLE patients have impaired endothelial function compared to healthy controls.
2. If SLE patients with type I IFN activity have endothelial dysfunction and platelet activation.

B.) Lipid dysregulation is seen in SLE and is thought to contribute to the elevated CVD risk. The vasculoprotective apolipoprotein M (apoM) is suggested to promote endothelial health and apoM plasma levels are demonstrated to decrease during inflammation. We aimed to investigated if plasma levels of apoM

1. Is decreased in SLE patients with active disease.
2. If SLE patients with decreased plasma apoM levels have impaired endothelial function.

C.) Polymorphonuclear leukocytes (PMNs) play a role in the pathogenesis of both SLE and atherosclerosis. The pro-inflammatory protein complex S100A8/A9 and S100A12, the calgranulins, are abundant cytosolic proteins in PMNs and are released upon PMN activation. The S100 proteins are not only markers of inflammation, but might play a role in the disease mechanisms of both SLE and CVD acting through receptor interaction on immune cells and endothelial cells. The aims were to investigate:

1. If serum calgranulin levels are elevated in SLE patients with increased disease activity, certain specific organ manifestations and presence of autoantibodies in SLE.
2. If increased serum levels of calgranulins are association with CVD in SLE

Patients and methods

Patients

Correct clinical assessment is crucial when diagnosing and evaluating SLE patients. Rheumatologists with expertise in SLE evaluation examined the SLE patients included in the current studies of this thesis. SLE patients at the Department of Rheumatology Skåne University Hospital, Lund were asked to participate in studies related to cardiovascular disease (paper III and patient group 2, paper IV). Pregnant or breastfeeding patients were excluded as well as patients with dementia, not able to fill in questionnaires and patients too sick to participate in the EndoPAT examination and/or to come to the clinic for research purposes. In patient group 1, paper IV, stored serum samples from SLE patients were collected with the aim to select samples from time points of active disease, representing a wide range of manifestations and SLEDAI-2K scores, including a number of patients with no disease activity. In paper II, paired serum samples from SLE patients were collected at time points of lower and higher disease activity. In paper I, serum samples from all SLE patients participating in a prospective follow up program for which a matching visit at the clinic at which the patient had low disease activity, were selected. In paper I and II, serum samples from time points of infection were excluded since infections affect S100A8/A9 and S100A12 serum concentrations to a large extent.

Disease activity

In paper I, II and in patient group 1 in paper IV, the SLEDAI-2K score was estimated retrospectively. The SLEDAI-2K is validated for retrospective use [406]. The SLE patients in paper III and patient group 2 in paper IV were investigated by a rheumatologist at inclusion of the study and disease activity was estimated by SLEDAI-2K.

Damage index

SLICC/ACR organ Damage Index (DI) is recorded prospectively in the SLE patients at the Department of Rheumatology, Skane University Hospital in Lund and these data were used for DI score in papers I-IV and verified retrospectively from medical records. If data were missing, DI was scored from medical records. Retrospective recording of DI has been evaluated[407].

Classification of glomerulonephritis in SLE

Glomerulonephritis (GN) in paper II was classified according to the World Health Organization (WHO) classification of renal biopsies in patients biopsied in 2006 and earlier. In patients biopsied in 2007 or later, GN was classified according to the International Society of Nephrology and Renal Pathology Society (ISN/RPS) classification. To get comparable biopsies, re-evaluation of the biopsies to ISN/RPS classification could be performed and that might be important in future studies for evaluation of S100A8/A9 serum levels in relation to GN classification and renal outcome.

Estimation of renal function

To determine renal function in a reliable way is most important to be able to decide dosage and type of medication to use and, in SLE patients, to evaluate renal organ damage after glomerulonephritis. Renal function is best described by the filtration rate in the glomeruli, glomerular filtration rate (GFR). Iohexol clearance is an accurate method to assess GFR, and used in research and clinical practice. GFR can also be estimated by different validated equations using the plasma creatinine- and /or cystatin C value, age, gender and in some cases ethnic group, height and weight. In paper II, estimated GFR was determined using the modification of diet in renal disease (MDRD) equation[408]. This equation includes age, gender, ethnic group, *e.g.* Caucasian or African, and plasma creatinine value. This validated method was used since we had access to all data needed to calculate GFR.

CVD definitions

Paper I and III

Cerebrovascular incident (CVI), acute myocardial infarction (AMI), deep venous thrombosis (DVT) (paper I and III), angina pectoris and claudicatio (paper I) were verified in medical records, ascertained by clinical assessment and defined by the SLICC/ACR Damage Index (DI) regardless of time point of SLE diagnosis. Thus, cardiovascular events that occurred before SLE diagnosis were also included. The following definitions defined by the SLICC/ACR-DI were used:

Cerebrovascular incident

Stroke with irreversible or partially reversible motor and/or sensory deficits including cerebral infarction or cerebral hemorrhage confirmed by computer tomography or magnetic resonance imaging.

Acute myocardial infarction

Acute myocardial infarction (AMI) confirmed by electrocardiography and rise in plasma creatine kinase, muscle and brain fraction (CKMB) or troponin T and/or coronary occlusion at coronary angiography.

Angina pectoris

Severe discomfort or pain over the sternum, anterior left chest and left arm. Electrocardiographic abnormalities.

Intermittent Claudication

Pain and weakness in the legs during walking that disappears at rest.

Deep vein thrombosis

Deep vein thrombosis confirmed by ultrasonography or venography and/or pulmonary embolism, confirmed by angiogram or radionuclelide lung scanning.

Assessment of endothelial function

Invasive investigation of coronary arteries

Endothelial function can be measured by an invasive method where acetyl choline is injected into the coronary artery and the responding change of coronary artery volume is detected by x ray imaging observing changes of artery diameter. In the

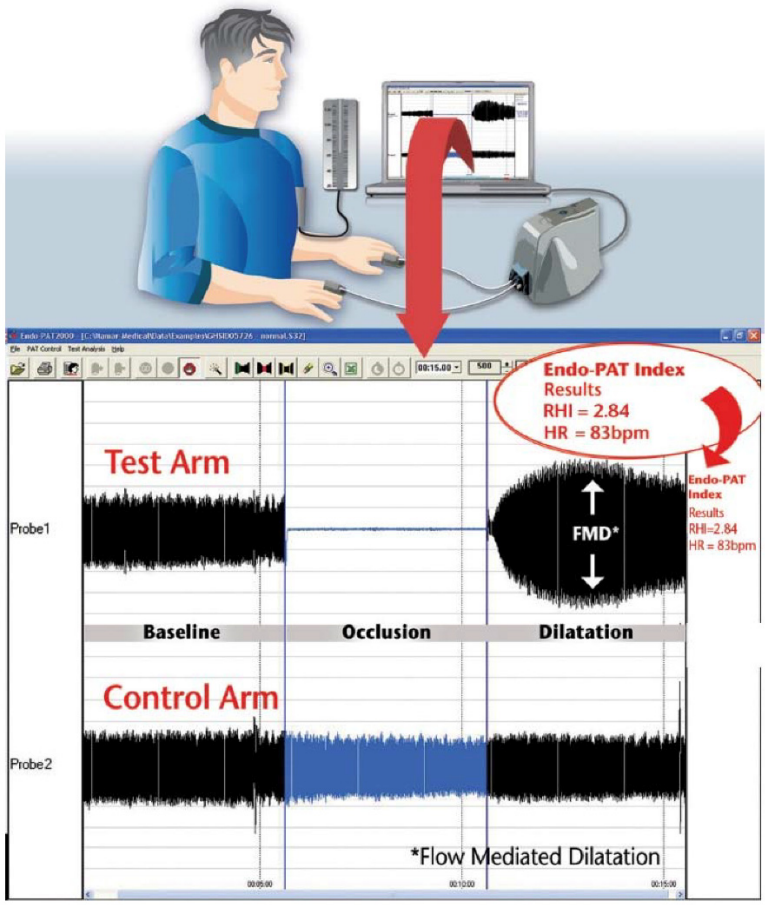
healthy endothelium of the artery an increased diameter is seen while decreased artery diameter indicate endothelial dysfunction. This technique is invasive and requires hospitalization and is not practical for use in CVD primary preventive risk estimation.

Flow mediated dilatation

In the early 1990s a technique named flow mediated dilatation (FMD) was developed. This is a non-invasive investigation in which changes in diameter of the brachial artery is estimated using an ultrasound imaging probe, after 5 minutes of supra systolic occlusion by a blood pressure cuff that is then released. A larger reactive hyperemic response with increased artery diameter as a response to the elevated pressure after cuff release, indicate normal endothelial function. This method requires an experienced sonographer and is operator-dependent.

EndoPAT assessment of endothelial function

In paper III, endothelial function is analysed with EndoPAT. Assessment of endothelial function with EndoPAT is non invasive and operator-independent. EndoPAT was tested against the invasive gold standard technique in 2004 in which there was a correlation between coronary vasoreactivity; coronary artery blood flow (CBF) in response to acetylcholine, and reactive hyperemia index in the finger arteries ($r=0.405$, $p<0.001$). Further, the average RHI value was lower in patients with coronary endothelial dysfunction compared to those with normal[409]. Peripheral Arterial Tone (PAT) measures tone changes in the finger artery beds. Through a sensor, the finger artery pulse volume is registered at 5 minutes rest, 5 minutes supra systolic pressure and after cuff release for 5 minutes to register the reactive hyperemic response (figure 4a). The pulse wave in a finger of the contralateral, non-occluded arm is registered as a control. Reactive hyperemia index (RHI) is the post-to-pre occlusion PAT signal ratio, normalized to the control arm. Results are automatically calculated. A normal hyperemic response is shown in figure 4A and endothelial dysfunction is shown in figure 4B, showing a graph from one of the SLE patients from paper III .



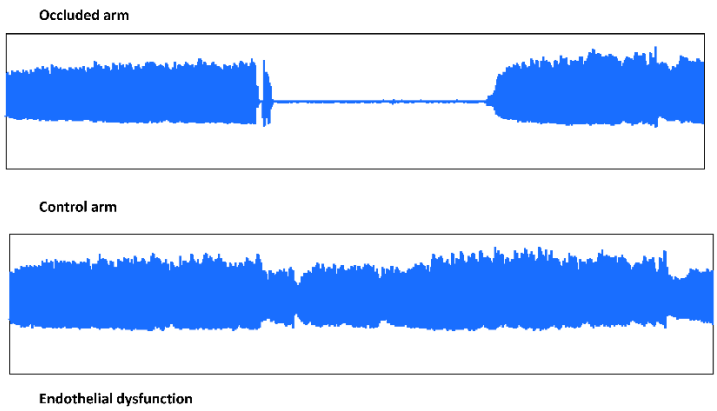


Figure 4A, 4B Assessment of endothelial dysfunction with EndoPAT
4A Normal endothelial function. 4B Endothelial dysfunction. *4A printed with permission from Itamar-medical*

EndoPAT assessment of arterial stiffness

As subjects were tested with EndoPAT 2000 the EndoPAT software calculated augmentation index as described previously[410]. When AI is analysed by EndoPAT, the magnitude and timing of the wave reflection in the digital pulse is considered[411]. EndoPAT arterial stiffness measures correlate well with AI measured with other devices[412].

To determine the cut-off for the augmentation index (AI), the residuals (differences in observed and expected AI) were calculated in healthy controls using a regression line for AI and age. Individuals with AI values above the expected value were classified as positive and individuals with AI values below the expected value were classified as negative. A positive AI value indicates a higher arterial stiffness than expected for age. Since the parameter AI is influenced by heart rate, it is standardized to a heart rate of 75 beats per minute (bpm), (AIx@75). In this presentation, (AIx@75) is referred to as AI.

Laboratory tests

Type I IFN analyses

Due to lack of sensitive commercial assays available to detect serum levels of IFNα we instead analysed type I IFN activity using functional assays as well as determining the expression of type I IFN regulated genes and protein, as depicted below.

Serum type I IFN activity

Type I IFN activity was assessed with a reporter cell assay as described previously with some modifications[413]. WISH cells were cultured with serum from SLE patients. If the serum contain type I IFNs, it will bind to the IFNα receptor (IFNAR) and induce transcription of type I IFN regulated genes. The WISH cells were then lysed and analysed for mRNA expression of three house keeping genes and six type I IFN-regulated genes using Luminex. The IFN score was calculated by the ratio between IFN-regulated genes and the house keeping genes. To achieve a relative expression of the type I IFN-regulated genes the ratio was compared with that in unstimulated WISH cells, cultured with medium.

PBMC type I IFN signature

PBMCs from the SLE patients were isolated and lysed and the type I IFN signature analysed, using the same Luminex assay as for serum IFN score. mRNA expression of three house keeping genes and six type I IFN regulated genes were analysed. The mRNA expression in the PBMCs was analysed and normalized to the expression of the house keeping genes. The sum of the IFN regulated genes was divided with the sum of the house keeping genes to achieve a relative expression of the type I IFN regulated genes.

G3BP

The interferon-inducible protein galectin-3-binding protein (G3BP) was measured with ELISA.

There was a good correlation between all three type I IFN assays (results in paper III).

S100A8/A9 ELISA

We used an in-house ELISA for assessment of serum S100A8/A9 in papers I and II. Plates were coated with a monoclonal antibody against S100A8/A9 (27E10) and incubated overnight. Serum from the SLE patients were added and incubated for 2 hours before biotinylated chicken polyclonal antibodies to S100A8/A9 were added and incubated overnight. In the next step alkaline phosphatase-labelled streptavidin was added and after incubation, bound streptavidin was visualized by adding disodium-p-nitrophenyl phosphate and the absorbance was measure at 405nm. The concentrations were calculated from titration curves obtained from one serum with known concentration.

Assay for serum induced phagocytosis of necrotic cell material

As mentioned above, Bohm et al describe a new LE cell test using flow cytometry in 2004. In studies from our group, we have used a similar method for assessing and quantifying PMNs with engulfed nuclear material by flow cytometry[414]. *In vitro,* this phagocytosis by PMNs of nuclear cell material is mediated by added SLE serum and is dependent of antibodies to nuclear material (e.g. anti-histone- and anti-dsDNA antibodies) as well as a functioning classical complement pathway[414].

In paper II, phagocytosis of necrotic cells (NC) by PMNs was measured by this modified LE cell test. In the schematic picture below, the assay for assessment and quantification of LE cells in patients with SLE, used in paper II is demonstrated.

Freshly heparinized blood from healthy donors is used to obtain PMNs and peripheral blood mononuclear cells (PBMCs). To obtain necrotic cell material (NCM), PBMCs are incubated for 10 minutes at 70° C and the NCM is stained with propidium iodide (PI). PMNs are stained with anti-CD45-FITC antibodies.

PI-labelled NCM were incubated with serum from SLE patients at room temperature (RT) for 20 minutes, allowing antibodies to bind to the NCM. Normal human serum (NHS) from a healthy donor was used as a negative control as well as NHS supplemented with normal mouse immunoglobulins. As a positive control, NHS supplemented with mouse-anti-histone antibodies was used. Anti-CD45-FITC-labelled PMNs were added and incubated for 15 minutes at 37° C to allow for uptake of the antibody-and complement-opsonized NCM. Cells were washed with PBS before analysed by flow cytometry. PMNs were identified based on forward and side scatter properties and by computerized gating. Phagocytosis was calculated as the percentage of cells positive for both CD45 and PI (figure 5).

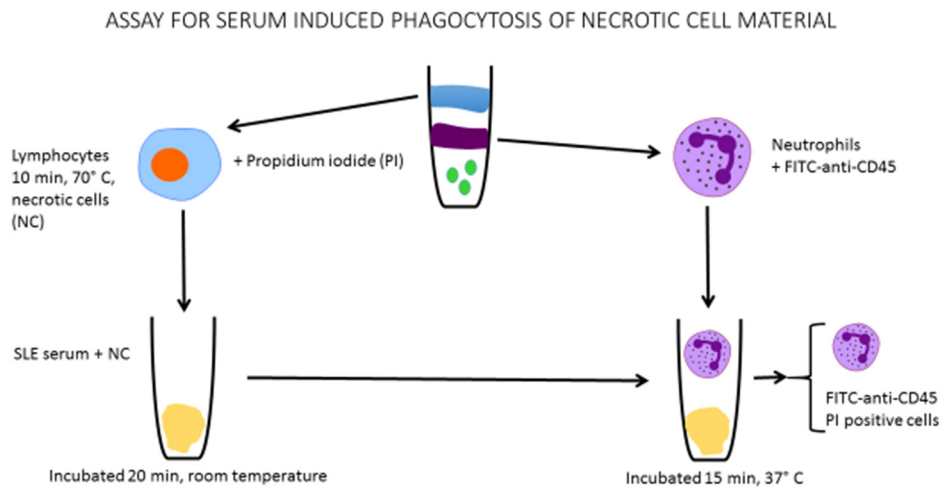


Figure 5 Assay for serum induced phagocytosis of necrotic cell material
Partially based on biodraw pictures

Endothelial microparticles analysed by flow cytometry

Endothelial microparticles (EMPs) are subcellular fragments that are generated during cell apoptosis, cellular activation including artery shear stress, pro-inflammatory or pro-thrombotic conditions and also during ordinary cellular differentiation. EMPs are markers of endothelial activation and their number and content mirror the state of the endothelium. Microparticles are defined as particles

less than 1 μm and they are measured by flow cytometry in platelet-poor plasma. Endothelial microparticles express CD146 and in paper III, the EMPs were labelled with murine monoclonal anti-CD146-FITC antibodies.

Statistics

When choosing the appropriate statistical methods the following aspects have been taken into account.

Description of data

Descriptive analyses have been used to describe observations and numerical data from the patient groups. For non-normally distributed data, non-parametric statistics have been used and data have been described by using median, range and quartiles. Normally distributed data are described with parametric statistics and described by using mean and standard deviation. For most of our analyses, including serum levels of S100A8/A9 and S100A12 in paper I and II, type I IFN parameters, markers of endothelial and platelet activation in paper III, and apoM levels in paper IV, non-parametric descriptive statistics was used.

Comparative analyses

To study differences between groups in continuous, non-normally distributed variables, the non-parametric Mann-Whitney U-test was used (papers I, II, III, IV). Categorical variables were analysed by Chi-square test (papers III and IV). For paired analyses, Wilcoxon signed rank test was used (paper II).

Correlation analyses

Correlations were assessed by Spearman rank correlation test, since this non-parametric test is robust when analysing correlations in non-normally distributed data (papers I, III and IV).

Multivariable logistic regression analysis estimated the associations between endothelial dysfunction and activation of the type I IFN system and platelets (papers III). This is also a robust test to assess associations, with possibility to adjust for confounding factors. This test can be used when predicting the probability of an outcome in relation to a number of prognostic variables.

Linear regression analysis was used to estimate the associations between EndoPAT RHI value and apoM concentration. When adjusting for CVD risk factors and treatment, multiple regression analyses was used (paper IV). To confirm that use of linear regression analysis was appropriate in this data set, the first step was to use the Shapiro-Wilk test to investigate the distribution and variation of the residuals, to establish a normal distribution of the residuals. Linear regression test is used when you want to predict an outcome dependent on one or several (multiple regression) factors.

Generalizability

If the results of our studies are applicable also to other SLE patient groups depends on how the patient groups are composed and of the characteristics of patients studied. The characteristics are usually presented in studies, as in the studies papers I, II, III and IV in table 1 and 2.

Results and discussion

Paper I

Increased serum levels of S100A8/A9 and S100A12 are associated with cardiovascular disease in patients with inactive systemic lupus erythematosus

The calgranulins, S100A8/A9 and S100A12 are markers of disease activity in SLE and thought to contribute to inflammation partly through interactions with their receptor RAGE. Ligand-RAGE binding induces intracellular signaling via mitogen-activated protein (MAP) kinase and nuclear factor kappa B (NF-KB) with subsequent production of pro-inflammatory cytokines[415]. As RAGE is expressed also on endothelial cells, calgranulin-binding might induce endothelial cell activation contributing to atherosclerosis development and subsequent CVD in SLE. This may be one explanation for the elevated CVD risk in SLE patient. Evaluation of the predictive value of S100A8/A9 for CVD in patients with an inflammatory disease is more complicated than in helathy individuals, because active disease can affect the calgranulin levels. Therefore, we analysed calgranulin serum levels in SLE patients (n=237) with low disease activity without infection, to minimize influence of flare on serum calgranulin concentrations. The aims were to find an association between calgranulin serum levels, CVD comorbidity and organ damage.

SLE patients with previous cardiovascular disease: *e.g.* cerebrovascular incidence (CVI) or acute myocardial infarction (AMI) had elevated calgranuline levels compared to SLE pateints with no previous CVD (Figure 6). This was not seen for venous thrombosis, consistent with a different underlying pathogenesis in arterial and venous thrombosis. Angina pectoris and claudication intermittens were not associated with elevated calgranulin levels which could be due to the low number of patients in these groups. Further, the diagnosis angina pectoris and claudicatio intermittens are to a large extent based on patient description of symptoms, as compared to CVI and AMI diagnosis which are based on several objective parameters. This may add some insecurity in the diagnostics that may affect our results.

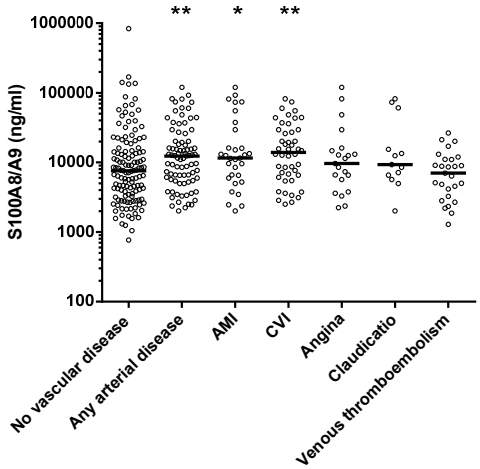


Figure 6 S100A8/A9 and cardiovascular disease
Serum levels of S100A8/A9 in SLE patients with and without cardiovascular comorbidity. Adapted from paper I.Reprinted with permission from Rheumatology Oxford, Tydén *et al*. Copyright © 2013.

SLE patients with any kind of organ damage, SLICC/ACR organ damage index (SDI) score ≥1, had elevated calgranulin levels compared to patients without organ damage. Presence of organ damage indicates a more severe disease. A hazard ratio of 1.48 for mortality has been demonstrated in the SLE cohort in Lund for every additional point scored in SDI[416]. Thus, elevated calgranulin levels could be indicators of severe disease and closer monitoring of these patients could be indicated.

SLE patients with no or low clinical disease activity are supposed to be in remission between periods of clinically active disease. However, the clinically inactive SLE patients in paper I (n=237) seem to have ongoing low grade inflammatory activity, in part mediated by PMNs because of the elevated calgranulin levels. Also when investigating patients with no clinical or serological activity and SLEDAI-2K score=0 (n=149), S100A8/A9 levels were elevated compared to healthy controls $p<0.0001$. Thus these patients could be undertreated with low grade inflammation contributing to organ damage including CVD and calgranulins could be markers of this need for treatment.

Patients with diabetes are known to have increased CVD risk and with dysregulated blood sugar they have elevated plasma levels of advanced glycation end products (AGE). AGE are proteins or lipids that are glycated when exposed to sugar and they are ligands to RAGE. Thus the elevated CVD risk in both diabetes and SLE could be mediated through RAGE-ligand interaction on endothelial cells

and might be a treatment target to prevent atherosclerosis development in these patients.

Blocking RAGE results in decrased inflammatory response in endothelial cells *in vitro* and *in vivo* [415]. Quinoline-3-carboxamide is an immune modulating substance that acts through binding to S100A9 and S100A12. Quinoline-3-carboxamide exerts its effect through T cell activation by APCs. Treatment with Quinoline-3-carboxamide has been found to reduce atherosclerotic plaque in mice[417], decrease disease activity in lupus prone mice and be well-tolerated in SLE patients during a clinical trial[418].

A limitation of this study (paper I) is that we did not have access to information about CVD risk factors: smoking habits, blood lipid profile, blood sugar concentrations, blood pressure and BMI. Therefore, we have not been able to adjust for CVD risk factors, which would have been of great value.

In conclusion elevated calgranulin levels may be markers of CVD and severe disease in SLE and might indicate patients who would benefit from more intense CVD primary preventive treatment and more accurate follow up. Furthermore, though not clinically active in their SLE, these patients might be subject to more intense SLE treatment to prevent CVD and organ damage

Paper II

Pro-inflammatory S100 proteins are associated with glomerulonephritis and anti-dsDNA antibodies in systemic lupus erythematosus

Serum levels of the calgranulins S100A8/A9 and S100A12 are elevated in a number of rheumatic and inflammatory diseases and suggested to participate in the pathogenesis of the diseases. We set up to analyse calgranulin serum levels as markers of disease activity and indicators of different kinds of organ involvement. Since the mechanisms behind the elevated serum levels of S100A8/A9 and S100A12 in SLE has been insufficiently investigated, we wanted to try the hypothesis that RNA and DNA-containing ICs, phagocytosed by PMNs in SLE, initiate an active release of calgranulins.

As part of a prospective follow up program at the Department of Rheumatology in Lund, we were able to identify stored frozen serum samples from 100 SLE patients matched for time points of low and high disease activity.

Our data demonstrated that S100A8/A9 and S100A12 were elevated in high disease activity as compared to low disease activity ($p<0.05$ and $p<0.01$, respectively). To our knowledge, S100A12 has not been demonstrated as a marker

of flare in SLE previously. SLE patients with glomerulonephritis, who had not started treatment with immunosuppressive drugs had elevated serum levels of S100A8/A9, but not S100A12 compared to patients with non-renal flare (Figure 7). Furthermore, serum levels of S100A8/A9 were reduced upon immunosuppressive treatment in 13 patients with glomerulonephritis and available follow up samples. Patients with skin rash, a mild manifestation of SLE, had lower S100A8/A9 levels compared to patients with active disease without skin rash. Those data might mirror the different mechanisms and involvement of neutrophils in glomerulonephritis as compared to rash.

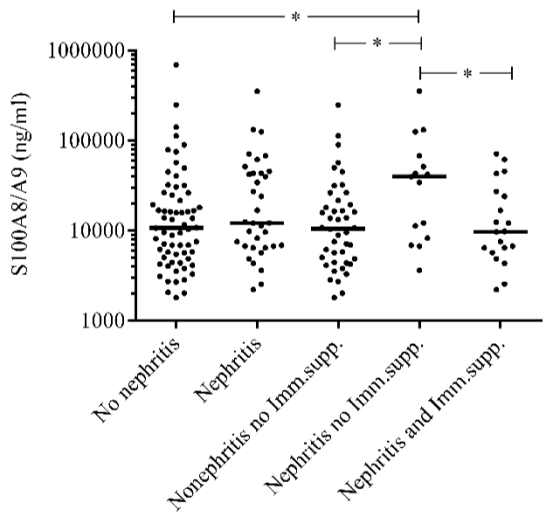


Figure 7 S100A8/A9 and glomerulonephritis
Serum levels of S100A8/A9 in SLE patients with and without glomerulonephritis who have or have not started treatment with immunosuppressive drugs. Adapted from paper II Lupus, Tydén et al.Copyright © 2016 by the Authors. Reprited by permission of SAGE Publications, Ltd.

In paper II, the number of patients with glomerulonephritis and available blood samples before start of treatment with immunosuppressive medication and at follow up visits after 3-4 and 6-10 months was relatively small. Further investigations involving a greater number of patients are needed to confirm if S100A8/A9 levels decrease after immunosuppressive treatment. Furthermore, among the patients with available follow-up samples, most patients in this study were partial renal responders to treatment (PRR) and the ACR-Renal Response Criteria is a rough measure of renal outcome. A larger number of patients with PRR, complete renal response as well as non responders to treatment should be examined in relation to S100A8/A9 serum concentrations and if possible with more refined measures of renal outcome, to evaluate the use of S100A8/A9 serum levels as markers to monitor treatment.

Increased serum-mediated phagocytosis of necrotic cells by neutrophils were associated with elevated S100A8/A9 serum levels both at time points of low and high disease activity.

We aimed to demonstrate that our results with elevated S100A8/A9 serum levels associated with increased phagocytosis of NC material by PMNs were due to active S100A8/A9 release from PMNs as a result of IC phagocytosis. However, due to technical difficulties, with isolated neutrophils spontaneously releasing marked levels of S100A8/A9, we were unable to confirm our hypothesis. Further, when we managed to isolate granulocytes that were not activated upon purification, they did not phagocyte ICs. Probably, granulocytes need to be activated to some extent to be able to phagocytose ICs. Further studies are needed to determine the underlying mechanisms related to release of S100A8/A9 in neutrophils and to whether this could be triggered upon phagocytosis of ICs.

To summarize, S100A8/A9 and S100A12 are markers of active disease in SLE and the different calgranulins do not follow the same pattern in the different organ manifestations. S100A8/A9 serum levels might decrease after immunosuppressive treatment. Our results are in line with the hypothesis that immunopathological events of the disease, increasing neutrophil activation (likely through engulfment of ICs), lead to release of S100A8/A9 that further enhance inflammation through downstream receptor interaction.

Paper III

Endothelial dysfunction is associated with activation of the type I interferon system and platelets in patients with systemic lupus erythematosus

The aims were to investigate endothelial function in SLE patients in a cross-sectional study of 148 SLE patients at the department of Rheumatology in Lund and compare with healthy controls. Further, as both platelet activation and IFNα are dysregulated in SLE and proposed to affect endothelial health, we assessed the associations between endothelial dysfunction, platelet activation and IFNα in the SLE patients.

We found that SLE patients with activation of the type I IFN system had lower reactive hyperemia index (RHI), indicating impaired endothelial function, compared with healthy controls (OR 2.61 95%CI 1.04-6.53 p=0.04).

The arterial stiffness marker; augmentation index (AI), was also analysed with EndoPAT[410,411]. Increased arterial stiffness predicts CVD and mortality in the

general population[419,420]. High augmentation index (AI) is a marker of arterial stiffness and increases with age.

Since the highest CVD risk increase in SLE patients is seen in the younger age group [227], we made subgroup analyses of young SLE patients when analysing AI by Endopat.

Young SLE patients, aged 20-45 years, had increased AI (standardised per 75 bpm) compared to age-and sex-matched controls, and the association remained after adjustment for CVD risk factors (age, gender, p-LDL, smoking, hypertension) (Table 1).

Table 1

Augmentation Index (AI) in SLE and healthy controls adjusted for CVD risk factors*

Tested groups	Augmentation index (AI)		
	OR	CI 95%	p
SLE (n=142) vs Healthy controls (n=78)	1.59	0.87-2.90	0.13
SLE IFN+ (n=30) vs Healthy controls (n=78)	2.01	0.74-5.47	0.17
SLE 20-45 (n=62) vs Healthy 18-45 (n=36)	**2.82**	**1.12-7.11**	**0.03**

* age, gender, smoking, hypertension, p-LDL concentration

No difference in AI (standardised per 75 bpm) was seen when comparing all SLE patients with healthy controls or SLE patients with high type I IFN activity (SLE IFN+) compared to healthy controls after adjusting for CVD risk factors.

We have thus demonstrated increased arterial stiffness in young SLE patients.

Importantly, the main differences were found in the younger age group, where it is more likely that we are studying impact of the SLE disease itself since traditional CVD risk factors influence the results to a higher degree in the older age groups.

In the SLE patients, an association between activation of the type I IFN system and endothelial activation, indicated by high sVCAM-1 and high EMPs was demonstrated (Table 2).

Table 2:

Correlations between endothelial activation and serum type I IFN activity in SLE.

Endothelial activation	sVCAM-1 high			EMP high		
	OR	CI 95%	p	OR	CI 95%	p
IFN activity[a]	**1.68**	**1.15-2.47**	**<0.01**	**1.40**	**1.04-1.88**	**0.03**

OR for indicators of endothelial activation; high sVCAM-1 and high EMP levels were calculated in SLE patients in relation to serum type I IFN activity, adjusted for CVD risk factors. EMP (endothelial microparticles). [a] adjusted for age, gender, smoking, hypertension, p-LDL concentration. Serum type I IFN activity was measured by a reporter gene expression assay (serum IFN score).

Endothelial activation, reflected by high sVCAM-1 levels and high EMP, was associated with platelet activation, measured as complement deposition on platelets (platelet C1q and C4d deposition) (Table 3).

Table 3:
Activated platelets in SLE patients with endothelial activation.

Endothelial activation	Platelet/C4d high			Platelet/C1q high		
	OR	95%CI	p	OR	95% CI	p
sVCAM-1 high[a]	**4.57**	**2.14-9.79**	**<0.01**	**4.10**	**1.09-15.38**	**0.04**
sVCAM-1 high[b]	**4.17**	**1.90-9.15**	**<0.01**	**4.15**	**1.07-16.18**	**0.04**
EMP high[a]	**3.64**	**1.16-11.38**	**0.03**	2.16	0.51-9.16	0.30
EMP high[b]	3.11	0.97-9.98	0.056	2.05	0.47-8.91	0.34

OR for markers of platelet activation; platelet-C4d and platelet C1q deposition was calculated in SLE patients with and without high sVCAM-1 and high EMP levels. Endothelial activation- and platelet activation values are dichotomous. EMP (endothelial microparticles), platelet-C4d/platelet-C1q (platelet-C4d/C1q deposition). [a] adjusted for age, gender, p-LDL concentration. [b] adjusted for age, gender, p-LDL and IFN activity.

Thus, our results are in line with the hypothesis that type I IFNs affect the endothelium leading to endothelial activation and dysfunction. Furthermore our data support that the endothelium also can be activated by platelets, a theory recently presented in a study where IL1β mediate EC activation through platelets.

The activated endothelium is suggested to interact with platelets and make them more adhesive to the vascular wall[421]. Furthermore, activated platelets interact with other immune cells with key functions in the SLE pathogenesis. Indeed, depletion of platelets or platelet inhibition with clopidogrel has been demonstrated to reduce disease activity and ameliorate nephritis in a lupus mouse model[144]. Platelet-pDCs interaction lead to increased IFN α production that might further affect the endothelium and platelet-monocyte/platelet-PMN aggregation could promote leukocyte migration over the vascular wall and mediate vascular inflammation. A vicious circle of inflammation, SLE disease activity and endothelial activation is formed (Figure 8).

A limitation of this study is the lack of mechanistic evidence of the observed associations. *In vitro* studies are needed to substantiate our findings. A strength of the study is the investigation of a relatively large group of SLE patients with, in general, low disease activity, and yet, indicators of endothelial and platelet activation was found that might be treated to decrease CVD risk. Prospective studies are needed to evaluate this.

In all, our findings support the theory, that different mechanisms in the SLE pathogenesis: type I IFNs and platelet activation, affect the endothelium and might contribute to development of endothelial dysfuntion. The activated endothelium itself is suggested to interfere with platelets and other immune cells, maintaining the inflammatory condition that is harmful for the cardiovascular system.

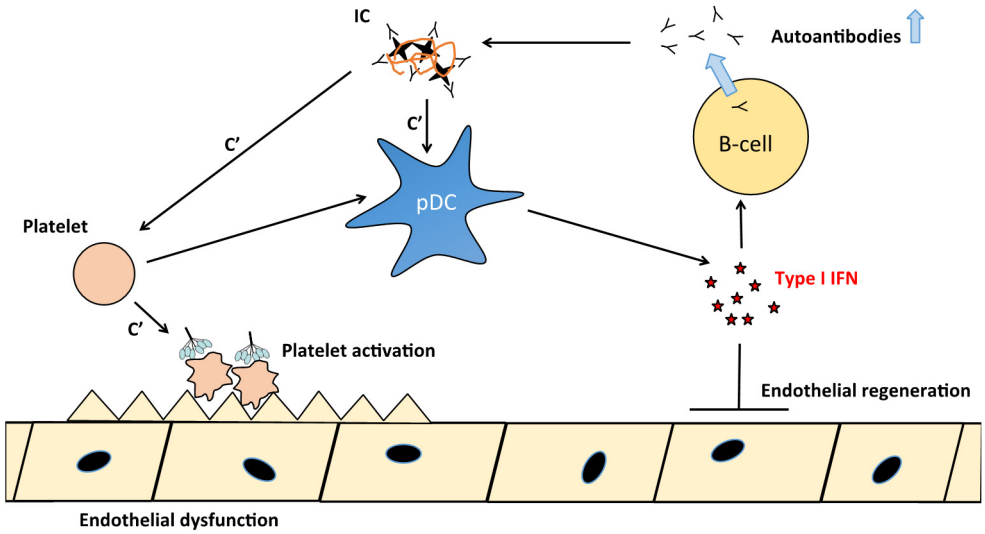


Figure 8 Endothelial dysfunction, activation of the type I IFN system and platelets
ICs stimulate pDCs to secrete type I IFN that contribute to endothelial dysfunction. Activated platelets interact with and adhere to the endothelium. Further, activated platelets affect the pDCs with subsequent type I IFN production.

Paper IV

Low plasma concentrations of Apolipoprotein M are associated with disease activity and endothelial dysfunction in systemic lupus erythematosus

Since systemic inflammation is known to lower plasma levels of the vasculoprotective apolipoprotein M (apoM), we hypothesized that SLE-related inflammation could affect apoM levels and that disease activity could increase the CVD risk in SLE by this mechanism. We aimed to analyse apoM levels in different disease manifestations in SLE.

ApoM is the carrier of sphingosine-1-phosphate (S1P), that through its receptors on endothelial cells promote endothelial barrier integrity and endothelial function[313]. We therefore wanted to investigate apoM levels in relation to endothelial function in SLE.

Even using the previously described SLE cohort with relatively low disease activity decreased plasma levels of apoM were seen in SLE patients as compared to healthy individuals ($p<0.01$). Patients with renal and skin involvement as well as presence of anti-dsDNA antibodies, had lower apoM levels compared to patients with other disease manifestations (Table 4). This difference might be caused by different mechanisms operating in the various disease manifestations affecting the apolipoprotein composition to varying degrees. The lower levels of apoM seen in patients with glomerulonephritis (GN) and anti-dsDNA antibodies fit well into the hypothesis that several different mechanisms contribute to the elevated CVD risk found in SLE patients with renal involvement[262]. Also in patients without SLE, the association between chronic kidney disease and CVD is well described[422].

Table 4.
Plasma levels of apoM in SLE patients with disease activity in different organ systems. Median plasma concentrations (μM) of apoM in SLE patients in patient group I (n=84) with (n+) and without (n-) different items in SLEDAI-2K

SLEDAI -2K items[a]	apoM	n+	apoM	n-	p
arthritis	0.82	20	0.70	64	0.38
Glomerulonephritis[b]	**0.55**	**21**	**0.76**	**63**	**<0.01**
rash	**0.55**	**29**	**0.80**	**55**	**<0.01**
low complement	0.67	37	0.75	46	0.14
anti-dsDNA antibodies	**0.54**	**24**	**0.78**	**60**	**0.01**
fever	0.59	9	0.73	75	0.32
thrombocytopenia	0.92	5	0.70	79	0.73
leukopenia	**0.50**	**13**	**0.76**	**71**	**<0.01**
alopecia	0.66	8	0.72	76	0.73

[a] Only items with n ≥5 included. [b] urinary casts, proteinuria, hematuria or pyuria

In young SLE patients aged 20-45 years (n =64), we found an association between lower apoM levels and endothelial dysfunction ($r=0.36$, $p=0.01$), also after adjusting for CVD risk factors, serum IFN activity, treatments with steroids, lipid lowering medication and antimalarials. This association was not seen when investigating the whole SLE group aged 20-82 years (n=148). The reason for this discrimination between groups could be that it is easier to detect changes in the endothelium related to SLE- related inflammation in the younger group. In younger patients, traditional CVD risk factors, have less impact on endothelial function.

In all, treatment of SLE disease activity could be beneficial for apoM levels and endothelial function in SLE patients. The S1P receptor on the endothelium, S1P or apoM could be future targets for treatment to preserve endothelial health.

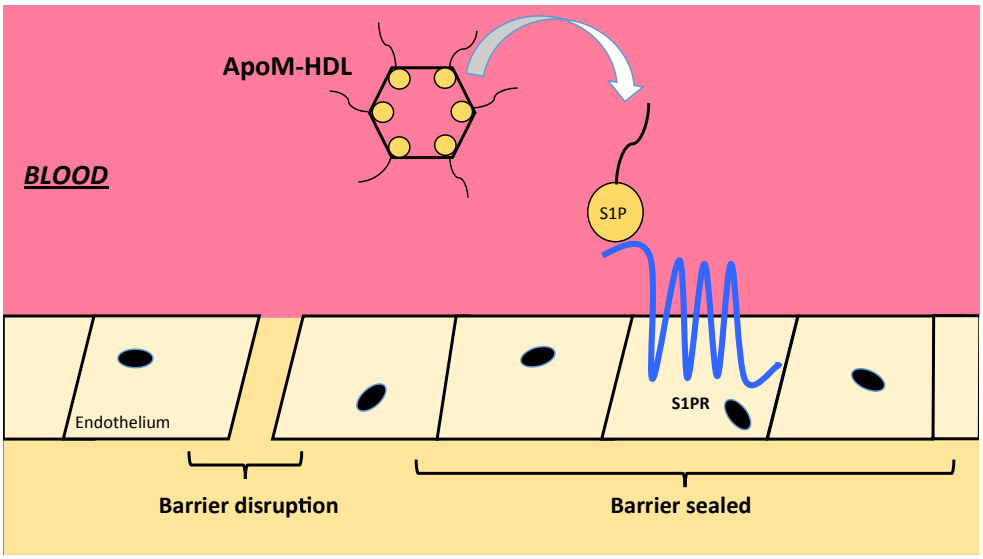


Figure 9 ApoM promotes endothelial function
ApoM-containing HDL transports S1P to its endothelial receptor. S1P-S1P-receptor interaction promotes endothelial barrier function.

General discussion

Even though SLE patients in a country like Sweden today are closely monitored and treatment is available, flares are seen in most patients over time and the chronic uncontrolled inflammation is thought to participate in development of irreversible organ damage, including cardiovascular disease (CVD) and venous thrombosis. Despite the increased CVD morbidity in SLE, there is no information on effectiveness of primary prevention and recommendations rely on vigilance concerning classical risk factors. Given that chronic inflammation over time has a great influence on organ damage, it is essential with close monitoring for disease activity as well as development of new biomarkers capturing immuno-pathologically relevant processes participating in disease progression and development of CVD in order to avoid organ damage and premature mortality, as well as identifying novel targets for treatment. Different mechanisms in the SLE pathogenesis are suggested to be CVD risk factors and involved in athersoclerosis and CVD development. Therefore it is crucial to understand the SLE disease mechanism in order to prevent CVD. The aims of this thesis were to investigate the associations between the immunopathology in SLE and atherosclerosis development that may lead to cardiovascular disease. We found marked endothelial dysfunction in SLE patients with type I IFN activation. The proposed role of type I IFN in development of endothelial dysfunction in SLE is well described. Since type I IFN is central in the SLE pathogenesis, affecting cells in both the innate and adaptive immune system and correlate with SLE disease activity, it is likely that the inflammatory state in SLE, to some extent driven by type I IFN, contribute to endothelial dysfunction. Treating inflammation by targeting type I IFN might improve endothelial function and reduce CVD risk in SLE patients. Several cells, including neutrophils and platelets, are crucial mediators in the pathogenesis of both SLE, endothelial dysfunction and atherosclerosis. Other molecules such as apoM, interact with the endothelium. As levels of apoM might be affected by SLE disease activity, an interplay between SLE disease and vascular health occurs. The calgranulins, S100A8/A9 and S100A12, are also examples of this interaction between SLE disease activity and the vasculature. The calgranulins, released from activated neutrophils, with serum levels increased in active SLE, mediate inflammation through their receptors on immune cells and drive further inflammation in SLE. Moreover, through receptor interaction on endothelial cells, mediating increased expression of adhesion

molecules and pro-inflammatory cytokines, affecting chemotaxis and cell migration, the calgranulins are also suggested to enhance atherosclerosis development pointing out PMNs as actors in CVD development in SLE.

Identification of risk patients is crucial to prevent CVD in SLE. New treatment strategies directed against platelets, neutrophils, type I IFNs and calprotectins, may target disease activity and thereby prevent endothelial dysfunction and atherosclerosis. Some of these therapies are already tested in humans an mouse models as discussed in this thesis.

In all, there is a great need to characterize the intricate interplay between the different inflammatory pathways operating in SLE, as discussed above, and how they contribute to CVD in order to identify novel prognostic markers as well as therapeutic targets to reduce the overall cardiovascular morbidity and mortality observed in SLE patients.

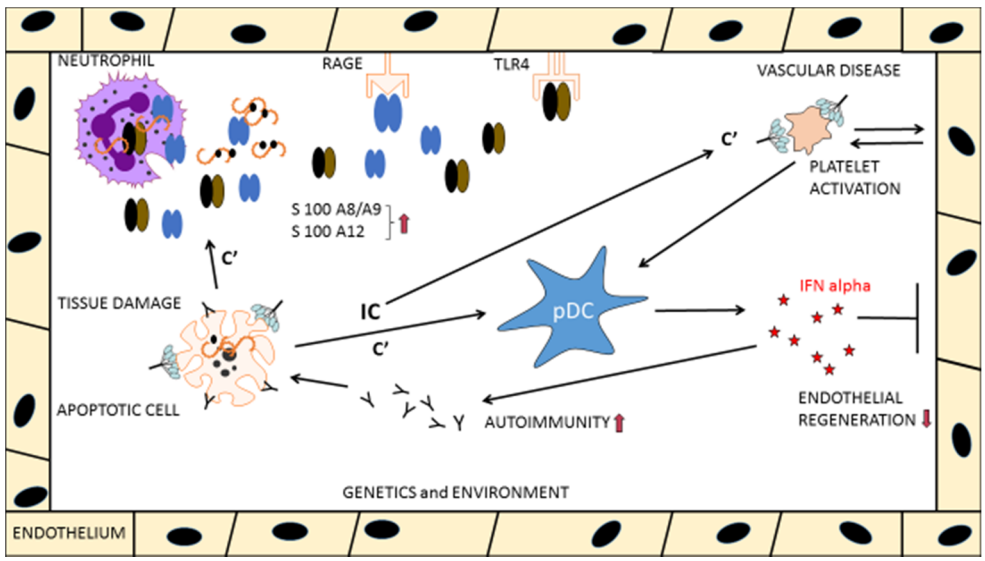


Figure 10 SLE and cardiovascular disease
The inflammatory pathways in SLE contribute to CVD development. Activated platelets interact with the endothelial cells and activate them. Platelets adhere to the activated endothelium. Platelets stimulate pDCs to type I IFN secretion. Type I IFN secretion is also induced by ICs, derived from autoantibodies that bind to apoptotic and necrotic cell material containing nucleic acid. Elevated type I IFN levels lead to endothelial activation and dysfunction. ICs can also activate neutrophils. S100A8/A9 and S100A12 mediate inflammation in the endothelium and through interaction with immune cells through their receptors TLR 4 and RAGE. *Partially based on biodraw pictures.*

Conclusions

In reference to the aims presented I would like to conclude:

- SLE patients with activated type I IFN system have impaired endothelial function.
- SLE patients with endothelial dysfunction have activated platelets. No direct correlation was found between platelet activation and type I IFN activity, indicating that platelet activation might be more a result of interaction with the dysfunctional endothelium. These results connect pathophysiological mechanisms in SLE with endothelial dysfunction and CVD development. Our data support the hypothesis that the endothelium, injured by type I IFN in SLE, may activate platelets and that platelet-endothelium interaction might contribute to CVD development. Our results suggest that assessing endothelial function, type I IFN signature and markers of platelet activation could be important when evaluating CVD risk in SLE.
- Plasma levels of apoM correlated inversely with disease activity in SLE, supporting that the inflammatory process lower apoM levels in these patients.
- The impaired endothelial function seen in young SLE patients with lower apoM levels is in line with the hypothesis that apoM plays a role in keeping endothelial health. Plasma apoM might be a marker of impaired endothelial function in young SLE patients
- Elevated serum levels of calgranulins in SLE patients indicate organ damage and CVD, suggesting a role for neutrophils in their development.
- Increased serum levels of calgranulins are markers of disease activity in SLE. The calgranulins are proposed to be involved in the SLE disease process, mediate inflammation and contribute to organ damage. Elevated levels of S100A8/A9 may be due to neutrophil activation and thus reflect neutrophil-dependent processes in SLE pathogenesis.

Future perspectives

In reference to the presented data I would like to discuss some possible future perspectives based on my studies.

- Assessment of endothelial function, type I IFN signature and markers of platelet activation could be important when evaluating CVD risk in SLE and this should be evaluated in prospective studies in larger SLE cohorts. As a pilot project, it would be of interest to investigate the SLE patients and healthy controls from paper III and evaluate the predictive value of reactive hyperemia index, augmentation index, platelet activation markers and type I IFN signature for cardiovascular events, organ damage and death.
- Future studies might elucidate whether endothelial-platelets interaction play a role in the increased CVD risk seen in SLE.
- Apolipoprotein M (apoM) has not been thoroughly investigated in SLE patients. Evaluation of apoM as a potential protective factor for cardiovascular events in SLE would be of interest. Further studies to confirm our findings of an inverse correlation between apoM levels and endothelial function in young SLE patients are warranted.
- The predictive potential of serum S100A8/A9 and S100A12 in relation to organ damage and CVD in SLE should be examined in a large number of SLE patients.
- I suggest further analyses of S100A8/A9 serum levels as biomarkers of glomerulonephritis(GN), response to treatment and relapse, in SLE patients. Investigation of serum S100A8/A9 in comparison to CRP and invasive measures such as renal biopsy should be performed.

Populärvetenskaplig sammanfattning

Systemisk lupus erythematosus (SLE) är en inflammatorisk, reumatisk systemsjukdom som kan engagera i stort sett samtliga organ i kroppen. Sjukdomsmanifestationerna kan vara relativt milda, med ledvärk och hudutslag, eller allvarliga med engagemang av njurar eller centrala nervsystemet. SLE är en autoimmun sjukdom, vilket innebär att immunförsvaret, som normalt ska skydda oss mot infektioner, angriper kroppsegna vävnader, vilket leder till inflammation. Antikroppar, som i normalfallet bildas mot angripande virus eller bakterier, bildas även mot kroppens egna proteiner, så kallade autoantikroppar.

Risken att insjukna i hjärt-kärlsjukdom är ca 2-10 gånger förhöjd hos SLE patienter jämfört med populationen i övrigt och den största relativa riskökningen ser man bland kvinnor under 45 års ålder. Denna riskökning beror på en kombination av så kallade traditionella kardiovaskulära riskfaktorer som rökning, högt blodtryck, och förhöjda blodfetter och riskfaktorer associerade med SLE sjukdomen, tex behandling med kortison, långvarig kronisk inflammation och förekomst av vissa autoantikroppar. Det finns även andra, ännu icke kartlagda orsaker till den ökade risken för hjärt-kärlsjukdom vid SLE.

I denna avhandling presenteras resultat från fyra studier som berör faktorer associerade med hjärt-kärlsjukdom vid SLE. De övergripande målen har varit att undersöka:

De proinflammatoriska proteinerna S100A8/A9 och S100A12 i förhållande till hjärt-kärlsjukdom, sjukdomsaktivitet och organmanifestationer vid SLE. Endotelfunktion hos SLE patienter i förhållande till typ I IFN-och trombocyt-aktivering. Nivåer i blodet av det kärlskyddande apolipoprotein M (apoM), i förhållande till sjukdomsaktivitet och endotelfunktion vid SLE.

De pro-inflammatoriska proteinerna S100A8/A9 och S100A12 (kalprotektiner) kan prediktera för hjärtinfarkt hos friska kvinnor och nivåer i blodet av dessa proteiner är förhöjda vid flera reumatiska sjukdomar, inklusive SLE. Man tror att dessa proteiner kan ha betydelse i sjukdomsmekanismen vid hjärt-kärlsjukdom, men även vid inflammatoriska sjukdomar som SLE. Vi visar att SLE patienter med genomgången hjärt-kärlsjukdom hade högre nivåer av S100A8/A9 och S100A12 i blodet, jämfört med hjärt-kärlfriska patienter. Även patienter med någon typ av organskada hade förhöjda protein- nivåer i blodet. Av detta kan man dra slutsatsen att S100A8/A9 och S100A12 kan vara markörer för allvarlig sjukdom och hjärt-kärlsjuklighet vid SLE.

Det finns ett behov av fler kliniskt användbara laboratoriemarkörer för sjukdomsaktivitet vid SLE. Vi underökte nivåer av S100A8/A9 och S100A12, i parade blodprover från SLE patienter med låg, respektive hög sjukdomsaktivitet. Nivån av proteinerna steg vid förhöjd sjukdomsaktivitet och detta var mest uttalat vid njurinflammation, där behandling ännu ej påbörjats, samt vid förekomst av en viss typ av autoantikroppar; anti-ds DNA antikroppar, som är associerade med njurinflammation. Hos patienter med hud inflammation, som är en mild manifestation av SLE, såg man lägre nivåer av S100A8/A9 jämfört med patienter med annan typ av organengagemang. Av detta kan man dra slutsatsen att S100A8/A9 och S100A12 kan fungera som markör för sjukdomsaktivitet vid SLE och indikera engagemang av vissa organ.

Typ I interferoner (IFN), är signalsubstanser som är centrala för att mediera inflammation vid SLE. Studier har visat att typ I IFN kan ha skadlig påverkan på endotelet, det innersta cellskiktet som klär hjärta och blodkärl. Endotelet har stor betydelse för att upprätthålla ett friskt hjärt-kärlsystem och nedsatt funktion kan vara första steget i utveckling av ateroskleros (åderförkalkning). Endotelet samspelar med blodplättarna, trombocyterna, som också har betydelse vid hjärt-kärlsjukdom. Vi visar att SLE patienter med pågående typ I IFN aktivitet hade sämre endotelfunktion än patienter utan IFN aktivering. Patienter med aktiverade trombocyter hade också försämrad endotelfunktion. Detta indikerar att endotel, skadat av IFN, aktiverar trombocyterna, en mekanism som skulle kunna förklara en del av den ökade risken för hjärt-kärlsjukdom vid SLE.

Hög-densitets-lipoprotein (HDL) transporterar kolesterol och fett i blodet, bort från cellerna till levern för vidare utsöndring från kroppen. HDL minskar även inflammation i blodkärlen och har visat sig skydda mot ateroskleros. Obalans mellan kärlskyddande och kärlskadliga lipoproteiner, har beskrivits vid SLE och kan bero på kronisk inflammation. Apolipoproteiner utgör en stor del av HDL, ett av dem är det kärlskyddande apolipoproteinet apoM, som enligt tidigare studier har visat sig vara gynnsamt för en god endotelfunktion. Vi fann sänkta nivåer av apoM vid SLE och att sänkta nivåer var relaterade till aktiv sjukdom, mest uttalat vid hud-och njurinflammation. Bland unga SLE patienter fann vi ett samband mellan låga apoM nivåer och endoteldysfunktion. Vi drar av detta slutsatsen att sänkta apoM nivåer vid SLE kan bero på sjukdomsaktivitet och vara en markör för endoteldysfunktion.

Sammanfattningsvis har vi föreslagit att mätning av kalprotektin i blod skulle kunna användas som markör för sjukdomsaktivitet, allvarlig sjukdom och förekomst av hjärt-kärlsjukdom vid SLE. Vi har även föreslagit att mätning av endotelfunktion, IFN-och trombocytaktivering samt nivåer av apoM, skulle kunna vara av värde vid värdering av risk för hjärt-kärlsjukdom hos SLE patienter.

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