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Studies on cardiovascular risk factors in Systemic Lupus Erythematosus

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To my Family

Original Papers and Manuscripts

This thesis is based on the following original papers and manuscripts, which will be referred to in the text by their Roman numerals

- I. **Gustafsson J**, Gunnarsson I, Börjesson O, Pettersson S, Möller S, Fei GZ, Elvin K, Simard JF, Hansson LO, Lundberg IE, Larsson A, Svenungsson E. Predictors of the first cardiovascular event in patients with systemic lupus erythematosus - a prospective cohort study. *Arthr Res Ther* 2009;11(6):R186
- II. **Gustafsson J**, Simard JF, Gunnarsson I, Elvin K, Lundberg IE, Hansson LO, Larsson A, Svenungsson E. Risk factors for cardiovascular mortality in patients with systemic lupus erythematosus, a prospective cohort study. Submitted.
- III. Svenungsson E, **Gustafsson J**, Leonard D, Sandling J, Gunnarsson I, Nordmark G, Jönsen A, Bengtsson AA, Sturfelt G, Rantapää-Dahlqvist S, Elvin K, Sundin U, Garnier S, Simard JF, Sigurdsson S, Padyukov L, Syvänen AC, Rönnblom L. A *STAT4* risk allele is associated with ischaemic cerebrovascular events and anti-phospholipid antibodies in systemic lupus erythematosus. *Ann Rheum Dis*. 2010 May;69(5):834-40
- IV. **Gustafsson J**, Gunnarsson I, Pettersson S, Zickert A, Vikerfors A, Hellbacher E, Möller S, Elvin K, Simard JF, Svenungsson E. Cigarette smoking and antiphospholipid antibodies in systemic lupus erythematosus. Manuscript.

Abstract

Systemic Lupus Erythematosus (SLE) is an autoimmune, inflammatory disease that mainly affects women. The prognosis of SLE has improved dramatically, but mortality rates are still higher than in the general population. With the improved general prognosis, cardiovascular disease (CVD) has emerged as a major cause of morbidity and mortality among SLE patients. Previous studies have demonstrated that the development of atherosclerosis is accelerated in SLE, and have identified a set of traditional and non-traditional risk factors that characterize SLE patients with CVD. Nevertheless, many unsolved issues with respect to SLE related CVD remain. The general aim of this thesis was to investigate risk factors for manifest CVD and for cardiovascular mortality (CVM) in SLE, with special focus on traditional risk factors, lupus phenotype, inflammatory and endothelial biomarkers, autoantibodies and genetic predisposition.

In the first paper, we prospectively studied traditional and non-traditional risk factors for the development of the first cardiovascular event (CVE) in 182 SLE patients with a follow-up time of 8 years. 24(13%) patients had a first event. We demonstrated that of the traditional risk factors, only age and smoking predicted the first CVE. Additionally, antiphospholipid antibodies (aPL), endothelial biomarkers, represented by soluble vascular cell adhesion molecule 1(sVCAM-1), and absence of thrombocytopenia were independent predictors of CVE. Thus, activation of the endothelium and the coagulation system are important features in SLE-related CVD and the importance to advocate smoking cessation among SLE patients is underscored

In the second paper, we prospectively investigated causes of mortality and risk factors for overall mortality and CVM in a cohort of 208 SLE patients, with a follow-up time of 12 years. We also evaluated Systematic coronary risk evaluation (SCORE, tool for evaluating the 10 year risk for cardiovascular death in the age span 40-65 years, based on traditional risk factors) in this population. Cystatin C, a sensitive measure of renal function, in addition to traditional and non-traditional risk factors, were evaluated as risk factors. 42 patients died, 48 % of which were due to CVM. Age, previous arterial events and high cystatin C levels were the strongest predictors for overall mortality and for CVM. After adjusting for these three variables, smoking, sVCAM-1 and high sensitivity C-reactive protein (hsCRP) predicted CVM. SCORE estimated 4 but we observed 9 cases of CVM, a non-significant difference. We conclude that except for smoking, traditional risk factors are less important than cystatin C, endothelial and inflammatory biomarkers as predictors of CVM in SLE patients.

In the third paper, we investigated whether a risk allele for SLE in the signal transducer and activator of transcription factor 4 gene (*STAT4*) was associated with vascular events or presence of antiphospholipid antibodies (aPL). A total of 578 unrelated SLE patients (424 from mid-Sweden and 154 from southern-Sweden) were included in a cross-sectional design. Occurrence of previous cardiovascular events and aPL were tabulated. Matched controls (N=651) were genotyped as a comparison. The results demonstrate that the *STAT4* risk allele was associated with ischemic cerebrovascular disease (ICVD), with a dose-dependent relationship between ICVD and number of risk alleles. The risk allele was furthermore associated with the presence of two or more aPLs, also in a dose-dependent manner. The association remained after adjustment for known traditional risk factors. We conclude that patients with the *STAT4* risk allele have an increased risk of ICVD. Our results imply that genetic predisposition is an important risk factor for ICVD in SLE patients, and that aPL may be one underlying mechanism.

In the fourth paper, we evaluated the potential association between smoking and aPL. 367 SLE patients were investigated in a cross-sectional study. Occurrence of aPL (anticardiolipin (aCL) IgG and IgM, anti- β_2 glycoprotein-1 IgG ($\alpha\beta_2$ GP1 IgG), lupus anticoagulant (LAC)) and smoking habits (never, ever, former, current) were tabulated. Never smoking was used as reference in all calculations. In multivariable models, adjusted for age, sex and age at disease onset, aCL and $\alpha\beta_2$ GP1 of the IgG isotype and LAC were associated with ever smoking, this association seemed to be driven mainly by the former smoking group. Our results demonstrate that smoking is associated with pro-thrombotic aPL in SLE patients, though we can not from this study draw firm conclusions about the temporal relationship between exposure to smoking and occurrence of aPL. Further studies are warranted to investigate the mechanisms behind these observations.

In prospective studies we have demonstrated that in particular smoking, systemic inflammation, endothelial activation and aPL are major risk factors for SLE related CVD and CVM. Furthermore, genetic predisposition, in our studies represented by a *STAT4* SLE risk allele, contributes to the high risk of ICVD and to the occurrence of aPL, a possible underlying pathogenic mechanism. Finally we demonstrate that smoking, known to have unfavorable effects on the immune system and to significantly increase cardiovascular risk in SLE patients, is also associated with pro-thrombotic aPL in patients with SLE. Thus in SLE smoking stands out as the most important of the traditional risk factors with potential influence also on lupus related risk factors such as aPL.

Abbreviations

ACR: American College of Rheumatology
aCL: anticardiolipin antibodies
ANA: Antinuclear Antibodies
a β_2 GP1: anti- β_2 glycoprotein 1
anti-dsDNA: antibodies to double stranded DNA
aSSA/B: anti-Sjögrens syndrome A/B
anti-oxLDL: antibodies against oxLDL
aPL: antiphospholipid antibodies
APS: antiphospholipid syndrome
C: complement component
CAC: coronary artery calcium
CAD: coronary artery disease
CVD: cardiovascular disease
CVE: cardiovascular event
CVM: cardiovascular mortality
FMD: flow mediated dilation
GFR: glomerular filtration rate
HDL: high density lipoprotein
HLA: humal leukocyte antigen
hsCRP: high sensitivity C- reactive protein
ICAM-1: inter cellular adhesion molecule 1
ICVD: ischaemic cerebrovascular disease
IFN: interferon
IHD: ischemic heart disease
IL: interleukin
IPVD: ischaemic peripheral vascular disease
IMT: intima media thickness
LAC: lupus anticoagulant
LDL: low density lipoprotein
Lp(a): Lipoprotein a
LPL: lipoprotein lipase
MBL: mannose binding lectin
MI: myocardial infarction

NK cell: natural killer cell
oxLDL: oxidized LDL
PON: paraoxonase
RNP: Ribonucleoprotein
SLAM: Systemic lupus activity measure
SLE: Systemic Lupus Erythematosus
SLICC: Systemic Lupus International Collaboration Clinics
SNP: single-nucleotide polymorphism
SMC: smooth muscle cell
SMR: Standardized mortality ratio
STAT4: Signal Transducer and activator of Transcription
TG: triglycerides
Th cell: T helper cell
TIA: transitoric ischaemic attack
TLR: toll like receptor
TNF: tumor necrosis factor
Treg: regulatory T cell
(s)VCAM-1: soluble Vascular Cell Adhesion Molecule 1
VLDL: very LDL
vWf: von Willebrand factor

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Systemic Lupus Erythematosus

Introduction and historical background

Systemic Lupus Erythematosus (SLE) is an autoimmune, inflammatory disease that can affect almost any organ. It is characterized by production of autoantibodies, immune complex formation and deposition that can lead to tissue injury. It manifests in many diverse clinical and serological patterns and has an unpredictable course.

Historically, the term lupus (latin for wolf) was used in the 12th century to describe all kinds of dermatological lesions. In 1845, von Hebra described the typical butterfly rash, the erythema, over the mid-face. A few years later, in 1852, the term lupus erythematosus was introduced by Cazenave(1). The systemic nature of the disease was recognized in 1872, when Kapozi described its disseminated features(2). This was further developed by Osler in the late 1890s, who introduced nephritis and vasculitis as manifestations of lupus(3). The modern period began in 1949 with the discovery of the LE cell (the lupus erythematosus cell) in the bone marrow of SLE patients(4). First recognized as pathognomonic for SLE, it has later been shown to be present in other autoimmune conditions as well. The development of immunofluorescent techniques in 1957 allowed the detection of antinuclear antibodies in clinical practice. This immunological test, further sharpened by the introduction of anti-DNA antibodies in 1957, are still important tools for diagnosing SLE.

Clinical features and classification criteria

SLE is a chronic inflammatory disorder, which has a varied clinical course in different patients. It can be mild, only affecting a limited number of organs, or dramatic with life-threatening involvement of multiple organs. Among the organs most often affected are joints, skin, kidneys, serous membranes, nervous system, blood elements, blood vessels and lungs. General symptoms like malaise, fever, and fatigue are all common.

Except for various clinical manifestations, lupus is characterized by production of an array of autoantibodies. In fact, over 100 autoantibodies have been described(5). Antinuclear antibodies (ANA) are the hallmark of SLE, over 90% of patients are ANA positive. Antibodies to double-stranded DNA (anti-dsDNA) and the Smith (Sm) antigen are highly specific for SLE. Other autoantibodies that are studied in clinical practice include Sjögrens syndrome A and B antibodies (aSSA, aSSB), anti-ribonuclein (aRNP) antibodies, nucleosome antibodies and antiphospholipid antibodies (aPL). Some of these antibodies are reported to be associated with flares or specific organ involvement, such as anti-dsDNA with nephropathy, aSSA/aSSB with keratoconjunctivitis sicca, and aRNP with Raynaud's phenomenon.

The complexity of SLE becomes evident by studying the classification criteria for SLE provided by the American College of Rheumatology (ACR)(Table 1). These criteria were first published in 1971 and revised by Tan 1982(6). They include 9 clinical and 2 immunological criteria, and according to them, a definite SLE diagnosis is present if the patient has four or more of the eleven criteria. In 1997, modifications were made, including aPL as a criterion(7). Implementation of this modification would change the population of SLE patients, however, the criteria adapted by Tan et al are still the most commonly used. The classification criteria are mainly made for research purposes, and are not diagnostic of SLE.

1. Malar rash	Fixed erythema, flat or raised, over the malar eminences
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging
3. Photosensitivity	Exposure to ultraviolet light causes rash
4. Oral ulcers	Includes oral and nasopharyngeal observed by a physician
5. Arthritis	Non-erosive arthritis involving peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	Pleuritis or pericarditis, documented by ECG or rub or evidence of effusion
7. Renal disorder	Proteinuria >0.5 g/d or 3+, or cellular casts
8. Neurologic disorder	Seizures or psychosis without other causes
9. Hematologic disorder	Hemolytic anemia or leukopenia (<4000/L) or lymphopenia (<1500/L) or thrombocytopenia (<100,000/L) in the absence of offending drugs
10. Immunologic disorder	Positive LE cell preparation or anti dsDNA or anti-Sm antibodies or false positive VLDR, detectable aPLs or LAC*
11. Anti-nuclear antibodies	An abnormal titer of ANA by immunofluorescence or an equivalent assay at any point in time in the absence of drugs known to induce ANAs

*If four of these criteria are present at any time during the course of the disease, a diagnosis of SLE can be made with 98% specificity and 97% sensitivity. Source: (6). * detectable aPL or LAC was added in 1997(7). Sm: the Smith antigen, LE: lupus erythematosus cell, VLDR: Veneral Disease Research Laboratory, ANA: anti-nuclear antibodies, aPLs: antiphospholipid antibodies, LAC: lupus anticoagulant activity*

Estimations of disease activity and damage

The course of SLE is variable and mostly unpredictable. Several instruments have been developed for the assessment of disease activity and clinical response to treatment. The most commonly used are SLEDAI (SLE Disease Activity Index; over the last ten days)(8), SLAM(Systemic Lupus Erythematosus Activity Measure; activity over the last month)(9), and BILAG (British Isles Lupus Assessment Group)(10). SLICC (Systemic Lupus International Collaboration Clinics) (11) is a well established damage index, demonstrated to be useful in evaluating long term outcome.

Epidemiology

SLE is a fairly rare disease, but incidence and prevalence figures are difficult to compare because epidemiological studies may differ by sampling and recruitment

methodologies used. Incidence rates of SLE range from approximately 1 to 10 per 100,000 person-years and prevalence generally range from 20 to 70 per 100,000(12). In a relatively recent study from southern Sweden, the prevalence was 68/100000 with an annual incidence of 4.8/100000(13). SLE predominately affects women of reproductive age, with a gender difference favouring women over men of 9:1(12). In the 1950s, the estimated 5-year survival in lupus was less than 50%(14) whereas recent studies report a 5-year survival over 90%(15, 16). Nevertheless, the mortality rate in SLE still exceeds that of the general population(17, 18). Death related to lupus activity and infections has decreased over time, but still contribute to mortality(19, 20), especially in developing countries(21, 22). However, cardiovascular mortality (CVM) has not declined(23) in SLE. A slight increased standardized mortality ratio (SMR) due to vascular diseases has been reported(24), and death from cardiovascular disease (CVD) accounts for between 6% and 76% in different studies(13, 25). (Table 2)

Reference	Study period	CVD%	SLE(active or endstage disease)%	Infection%	Malignancy%	SMR	Country
<i>Dubois et al 1974(26)</i>	1950-1963	9	57	15	4		USA
<i>Urowitz et al 1976(27)</i>	1970-1974	45	18	36			Canada
<i>Wallace et al 1981(28)</i>	1950-1980	18	34	21	3		USA
<i>Reveille et al 1990</i>	1975-1984	11	12	47	7		USA
<i>Abu-Shakra et al 1995(29)</i>	1970-1993	26	16	32	6.5	4.92	Canada
<i>Ward et al 1995(30)</i>	1969-1983	6	34	22	6		USA
<i>Stahl-Hallengren et al 1999(13)</i>	1981-1991	76	24	6	6		Sweden
<i>Jacobsen et al 1999(31)</i>	1975-1995	20	29	20	7	4.6	Denmark
<i>Moss et al 2002(25)</i>	1978-2000	17	15	17	20	4.0	United Kingdom
<i>Alamanos et al 2003(32)</i>	1981-2001	24	10	10	19	1.5	Greece
<i>Cervera et al 2003(33)</i>	1990-2000	19	26.5	25	5.9		Multicenter
<i>Björnådal et al 2004(23)</i>	1964-1995	42	21	1	12	3.63	Sweden
<i>Bernatsky et al 2006(24)</i>	1958-2001	12		5	9	2.4	Multicenter
<i>Chambers et al 2009(20)</i>	1978-2004	20		25	18		United Kingdom

*SMR: standardized mortality ratio. Empty cells = not evaluated

Ethiopatogenesis

The immune system, overview

The immune system is divided into different lines of defense. The first line is physical barriers, like the skin and mucosal membranes.

The second line of defense is the innate immune system, which comprises the cells and mechanisms that defend the host from infection by other organisms in a non-specific manner. The innate immune system is fast, but not specific, and does not confer long-lasting or protective immunity. The functions of the innate immune system include recruiting immune cells to sites of infection, through the production of chemical factors called cytokines. By activation of the complement cascade, bacteria are identified and opsonized (bound to), and cells (mostly macrophages) are activated to promote clearance of dead cells, microorganisms and debris. Finally, the innate immune system also activates the adaptive immune system.

The adaptive, or specific, immune system, is the third line of defense. It is the most specialized form of defense, and exists only in vertebrates. It has the ability to recognize almost an infinite diversity of specific antigens, through the process of antigen presentation. It is also characterized by immunological memory, in which each pathogen is “remembered”. The memory cells can be called upon to quickly eliminate a pathogen should subsequent reinfections occur. The main cells involved are the B- and T- lymphocytes. Clones of these cells have specificity for a certain antigen, and when they encounter this antigen, they amplify through clonal expansion, giving an effective defense against the invader. The B cells play a major role in the humoral immune response, whereas T-cells are intimately involved in cell-mediated immune responses.

The creation of antibodies, the humoral immune response, takes place in B-cells. Antibodies are soluble B-cell receptors, which upon encountering their antigen tag it and thereby facilitating uptake by macrophages or destruction by the complement system.

T-cells constitute the cellular immune response. They recognize an antigen when presented to them by dendritic cells, B-cells or macrophages. The T-cells are divided into two main subgroups, T-helper (CD4+) cells, and cytotoxic (CD8+) T-cells. The T-helper cells are further divided into Th1 and Th2 helper cells. The CD8+ cells induce death in cells that are infected with microorganisms.

T helper cells produce large amounts of cytokines, different depending on subtype, with the purpose of signaling, recruitment, activation, and differentiation of other cells involved in the immunological response. Th-1 cells produce interferon- γ (IFN- γ), interleukin 2(IL-2), interleukin 12(IL-12) and also tumor necrosis factor- α (TNF α), the latter although mainly derived from macrophages. Th-1 cells activate macrophages and promote a local inflammatory response. IFN- γ primes macrophages and influences B-cells during class switching to produce IgG3 antibodies, good at opsonizing viruses and bacteria and at fixing complement. TNF- α activates macrophages and natural killer cells (NK cells), and IL-2 is a growth factor for cytotoxic T-cells and NK cells. Thus, Th-1 cells intensify the cellular immune system by stimulating the phagocytic effect of macrophages, the proliferation of cytotoxic T-cells, and also the production of opsonizing antibodies. Th-2 cells secrete interleukin 4, 5 and 10 (IL-4, 5, 10), which are important for the humoral immune system. These cytokines activate B-cells, and they

promote proliferation into plasma cells, antibody production and Ig class switching .

The complement system

The complement system is made up of a large number of plasma proteins, among which the activation of one component activates the next in a cascade reaction. It can be activated through three pathways; the classical pathway, the mannose-binding lectin (MBL) pathway, and the alternative pathway. The early events of these three pathways involve a series of cleavage reactions resulting in the formation of C3 convertase, C4bC2a in the classical and C3bBb in the alternative pathway. This leads to cleavage of complement component C3 into C3a and C3b. C3b is then involved in a process in which the terminal components C5b, C6, C7, C8 and C9 form the membrane attack complex (MAC) which can lyse pathogens and certain cells. C3b and other cleavage products can also serve as opsonines on bacteria and immune complexes, facilitating clearance by macrophages. Other products of the complement system, C5a, C3a and C4a, mediate inflammation as they recruit and activate inflammatory cells. The classical pathway is mainly initiated by interaction of C1q with antibodies (IgG and IgM) in immune complexes and is the main effector of antibody-mediated immunity. The lectin pathway is triggered by the binding of MBL (in combination with the protein MASP) to certain carbohydrates, for example on bacteria. Finally, the alternative pathway is activated without any specific activator by spontaneous hydrolysis of C3 followed by interaction with factors B and D. Complement regulatory proteins tightly regulate complement activation, preventing both depletion of complement proteins and limiting complement-mediated host-cell damage.

The immune system in SLE

The autoimmune character of SLE suggests that the immune system is reacting against self. Multiple immunologic aberrations have been found in lupus, leaving little doubt that there is a fundamental derangement of immune homeostasis.

An overall concept of SLE pathogenesis is impeded clearance of cellular debris and apoptotic cells(34, 35), and failure to deplete autoreactive B-cells(36). Accelerated apoptosis of circulating lymphocytes and/or impaired clearance of apoptotic bodies may increase the amount of nuclear antigens presented to T lymphocytes. This can lead to production of autoreactive B-cells and autoantibodies, the latter a cardinal feature of SLE. Autoantibodies may induce damage by several mechanisms. Antibodies directed against cell surface membranes (e.g. erythrocytes, platelets), eliminate their target through complement-mediated lysis or by enhancing phagocytosis by macrophages. Another mechanism of antibody mediated tissue injury is deposition of immune complexes in tissues. This can be demonstrated by studying the microscopic findings in SLE nephritis and cutan lupus erythematosus, where these complexes often are abundant. A third possible mechanism for autoantibody mediated damage is by antibody dependent cellular cytotoxicity.

It is widely accepted that the activation of the classical complement pathway by immune complexes is an important contributor to organ damage in SLE. It can thus seem as a paradox that deficiencies within the complement system are strong susceptibility factors for development of SLE. However, the physiological activity of the early components of the complement pathway, such as opsonization and the removal of immune complexes and debris from the circulation, may play a role in host protection. Complement deficiencies/dysfunction, often present in SLE, can be

demonstrated by the impaired handling of immune complexes and decreased removal of apoptotic cells(37). Also, failure to deplete autoreactive B cells probably also involves the complement system, as complement is needed for the elimination of self-reactive lymphocytes during the maturation of the immune system(38). Furthermore, complement are probably also involved in cytokine regulation. It was recently reported that C1q can inhibit IFN- α production, suggesting an explanation for the up-regulation of the IFN system in SLE(39).

Interferon- α (IFN- α), a type I interferon, is an important cytokine involved in many immunological reactions including anti-viral defenses. It has been known for many years that patients with SLE have increased levels of IFN- α , and that these levels correlate to both disease activity and severity. There is also a correlation between IFN- α levels and markers of immune activation in SLE, e.g., anti-dsDNA titers, IL-10, and degree of complement activation. Clinically, high levels of IFN- α are associated with certain disease manifestations, such as fever, skin rash and leukopenia. The observation that IFN- α treated patients with non-autoimmune disorders can develop an SLE like syndrome further supports the role of IFN- α in the development of SLE(40, 41).

Many SLE patients show a pattern of IFN- α -inducible gene expression, the so-called "IFN signature". A type I IFN-signature was recently found in platelets from SLE patients, that was highly associated with a history vascular events(42).

Genetics

SLE has long been appreciated to arise from both genetic and environmental factors. Evidence for the genetic contribution for development of the disease come from the observation of familial aggregation and increased concordance in monozygotic twins(43). The patterns of inheritance are complex, however, and it is generally thought that variations in a number of genes are involved, each contributing a small amount to the overall genetic risk(44).

The effects from individual genetic risk factors for the development of SLE are relatively weak with a few exceptions. In particular, deficiencies in components of the classical pathway are associated with SLE(45). C1q deficiency is an extremely rare autosomal recessive complement disorder with only around 40 cases reported worldwide, associated with a very high risk of SLE or lupus like illness. Over 90% of cases develop disease(46). Deficiencies in C1r and C1s, also very rare, are associated with SLE in 68% of cases(47). The most frequently occurring homozygous complement deficiency in humans is that of complement component 2 (C2), 33% of these individuals develop SLE(48). Complete C4 deficiency is extremely rare, whereas heterozygous C4 deficiency with C4A or C4B-null alleles is more common. Total C4 deficiency and the presence of null alleles, especially for C4A, is associated with SLE(49). Recently, Yang et al also demonstrated a dose-dependent phenomenon of increased SLE risk at low C4 gene copy number. Protection against disease susceptibility is observed at high gene copy number(50).

For a long time, the human leukocyte antigen (HLA) system was the only known genetic region associated with lupus. The HLA region is located on chromosome 6 and contains hundreds of genes, many of which are important in the immune system and in autoimmunity. The HLA is divided into three clusters, class I, II and III. Class I includes HLA- A, -B and -C, and the classical class I gene products presents antigens to CD8+ cytotoxic T cells and are involved in NK mediated cell death. Class II includes HLA-DR, -DQ, DP and encodes proteins that present antigens to CD4+ T-helper cells.

Class III is somewhat different, encoding for immune proteins e.g. complement factors and cytokines.

The HLA region is highly polymorphic and it has been associated with most autoimmune, inflammatory and infectious diseases. There is also a high degree of linkage disequilibrium across the region, making it difficult to localize the specific genetic signal since long stretches of DNA are inherited together as haplotypes of alleles.

HLA class II haplotypes involving the HLA-DRB1 and HLA-DRQ1 loci have been described for their association with SLE, and in particular, haplotypes bearing the DRB1*1501/DQB1*0602 (DR2) and DRB1*0301/DQB1 (DR3) alleles have been associated with increased risk(51, 52). Also, genes within the HLA class III region, particularly the TNF- α (53), C2 and C4 gene loci (discussed above) have been in focus for research addressing the development of lupus

Although the HLA region to a large extent contributes to the genetic risk of SLE, other genes also play a role. For example, Fc receptors for immunoglobulin G, important for the clearance of immune complexes, has been implicated as risk genes(54). Interferon regulatory factor (IRF)5(55, 56), *ITGAM/ITGAX* (57) and *PTPN22* (58), also a strong genetic risk factor for RA(59), are other genetic risk factors.

STAT4 has reliably been demonstrated to be a strong genetic susceptibility factor for SLE(57, 60). STAT 4 transmits signals from the receptors for type I interferon, IL-12 and IL-23. STAT4 is a latent cytosolic factor that after activation by cytokines, is phosphorylated and accumulates in the nucleus. Activated STAT4 stimulates transcription of specific genes including IFN γ , a key indicator of T-cell differentiation into Th1 cells(61). STAT4 has also been implicated in the differentiation of Th17 cells, a rather new entity of T cells(62). Dependent on IL-23, proinflammatory Th17 cells can play an important role in chronic inflammatory disorders(63). The *STAT4* risk alleles are associated with a more severe SLE phenotype characterised by younger age at disease onset, higher frequency of nephritis and presence of anti-dsDNA (64, 65).

Sex hormones

That sex hormones are important for the development of lupus, and for other autoimmune diseases, is suggested by the female predominance(66). Hormones may have multiple influences on the regulation of the immune system. For example, modulation of cell growth and apoptosis, and influence on immunoglobulin production. Generally, steroid hormones are implicated in the immune response with estrogens as enhancers at least of the humoral immunity and androgens and progesterone (and glucocorticoids) as natural immunosuppressants(67). Potential hormonal risk factors studied for the development of lupus are age at menarche, oral contraceptives, breastfeeding, menopausal status and postmenopausal hormones(68). Treatment with dihydroepiandrosterone(DHEA), an androgen, has in some studies been shown to reduce disease activity and flares(69).

Environmental factors

Cutaneous photosensitivity is a well-known phenomenon in SLE and exposure to sunlight has been described as a trigger of SLE flares(70). Dietary constituents are of interest as aetiological risk factors for SLE. Alfaalfa sprouts have been shown to induce lupus-like disease(71). Lower levels of vitamin D have been observed in SLE, but it is not clear whether this has a causative effect or is a result of the disease(72). Also, certain drugs are able to induce a lupus-like syndrome, such as D-penicillamine and

sulphasalazine(73, 74). Alcohol might have a protective effect, although the results between studies are conflicting(75, 76). Other environmental factors which have a potential role in SLE pathogenesis are socioeconomic status, environmental contaminants, cosmetics, vaccinations and stress(68).

Smoking

Smoking is a well-established risk factor for the development of rheumatoid arthritis (RA). In RA, former, passive and current smokers are at increased risk (Costenbader 2006). Also, duration and intensity are of importance (Criswell 2002). Smokers seem to have a more severe form of RA than non-smokers (Goodson 2004, Stolt P 2003).

Smoking was first demonstrated to be a risk factor mainly for rheumatoid factor (RF)-positive RA, and the disease risk was greatly enhanced in individuals carrying certain major histocompatibility antigens, more specifically the shared epitope of HLA-DRB1: HLA-DRB1 SE(77). Later it has been demonstrated that both smoking and HLA-DRB1 SE are riskfactors exclusively for anti-citrullinated protein antibody (ACPA) positive RA, and that HLA-DRB1 SE is linked to the presence of anti-citrulline immunity(78). Evidence for the hypothesis that smoking is able to induce autoantibodies in genetically predisposed patients was recently further strengthened by the observation that smoking appears to be associated with an increased risk of anti-Jo-1 positivity in HLADRB103-positive myositis patients(79).

Current smoking is associated with a small but significant increased risk of SLE. In a metaanalysis by Costenbader et al, the odds ratio (OR) was estimated to 1.5 for current vs never smokers(80). Data are scarce concerning whether previous smoking or only current smoking is a risk factor and whether there is a dose-response relationship between smoking and risk for SLE. Some studies indicate that current smokers have more serious cutaneous involvement(81, 82) and that there is a relationship between smoking and more severe SLE in terms of serositis and renal failure(83, 84).

Few studies have been concerned with putative mechanisms by which smoking might trigger disease. Recently, though, one study was able to demonstrate an association not only between smoking and SLE, but also between smoking and occurrence of anti-dsDNA(85). This finding parallels the relationship between smoking and antibodies in RA and myositis. In SLE, however, no study has yet addressed the gene-environment interaction between smoking and genes that confer susceptibility to SLE. A study that contradicts the results above is the work by Rubin et al(86). They found that anti-dsDNA IgG were higher in former smokers than current and never smokers. They hypothesized that smoking might suppress IgG antibody production, and that after smoking cessation, there is an exacerbation of humoral autoimmunity. Also, cigarette smoke might also influence the effectiveness of medications; this has been demonstrated for hydroxychloroquine(87).

The effects of smoking on the immune system are complex and not fully understood. It may contribute to autoimmunity through several mechanisms. It can cause tissue damage and increased apoptosis through generation of free radicals and metalloproteinases, induce inflammation, have an immunosuppressive effect and finally, it has antiestrogenic effects(88).

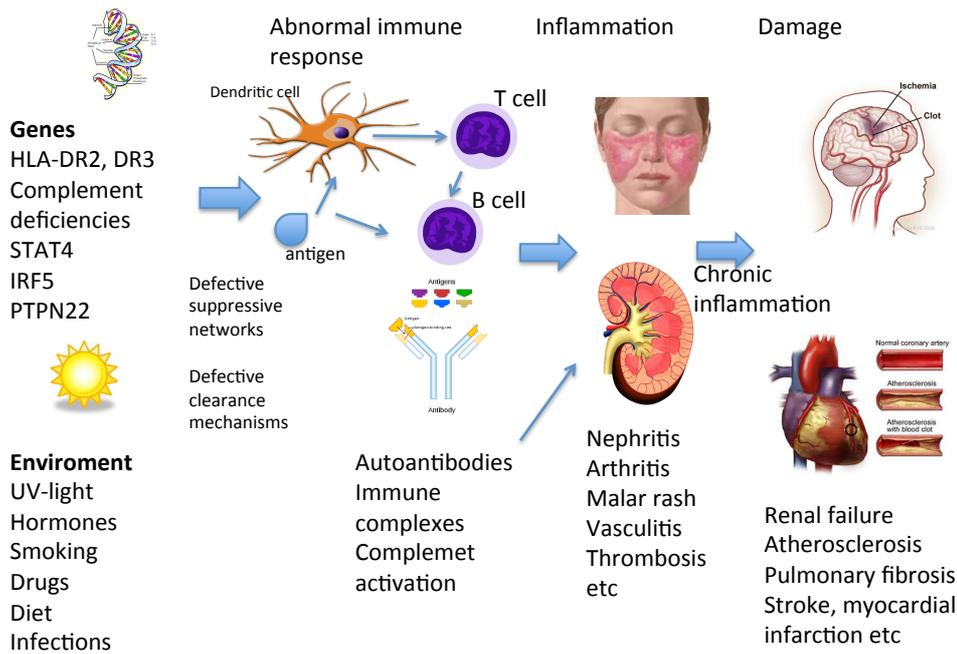


Figure 1. A possible model for the etiopathogenesis of SLE

Cardiovascular disease

CVD is the leading cause of mortality in the world. In Europe, CVD accounts for 49% of all deaths(89). In Sweden, approximately 40% of all deaths are due to CVD(90) Men are affected earlier and more often than women. CVD is rare before the age of 55 in women but at menopause there is a steep three to four-fold increased risk of CVD(91). Mean age for myocardial infarction in Sweden is 69-70 years for men, and 74-75 years for women(92). Although the incidence rates of CVD have been reported to be decreasing in the western world, cardiovascular health care is still a large challenge, especially in low- and middle-income countries.

Anatomy and physiology of the artery

The wall of large arteries consists of three layers, the intima, the media and the adventitia. The intima is the innermost layer, and consists of the endothelium, a sub-endothelial layer of connective tissue and an internal elastic membrane. The media consists of smooth muscle cells and collagen fibres. Its principal task is to regulate dilatation and constriction of the artery, and to control blood pressure. The adventitia is the outer layer consisting of fibroblasts, collagen and elastic fibres. In larger vessels there are also nerves, and blood supply (vasa vasorum). The endothelium is the continuous monolayer of cells that comes into direct contact with the blood. Its structure and functional integrity are important in the maintenance of the vessel wall and circulatory function, but the endothelium is by no means inert. As a barrier, the endothelium is semipermeable and regulates the transfer of small and large molecules.

Endothelial cells are dynamic and have both metabolic and synthetic functions. They exert significant autocrine, paracrine and endocrine actions and influence smooth muscle cells, platelets and peripheral leucocytes. The endothelium is involved in the production of growth factors, matrix products and inflammatory mediators. It has an important role in lipid metabolism. It has both antithrombotic and procoagulant functions, thereby maintaining blood haemostasis. It is also important in regulating vascular tone by secreting vasoactive substances. In this manner, the endothelium has emerged as an important mediator in several disease states, including infections, hypertension, auto-immune diseases and atherosclerosis(93).

Atherosclerosis- the main cause of CVD

Atherosclerosis is considered to be the main cause of CVD, and it affects all humans to a varying degree. Mainly, atherosclerosis is located to elastic and muscular arteries, with aorta and its branches, coronary arteries and cerebral arteries being primarily affected. The atherosclerotic lesion is located in the intima, between the endothelium and the smooth muscle cell layer. The process of atherogenesis begins in early childhood with the development of the "fatty streak"(94). Subsequently, the fatty streaks transform into fibromuscular plaques, consisting of a fibro-fatty core accumulation covered by a fibromuscular cap. The cap is formed by smooth muscle cells from the media, which have infiltrated the intima and have the function of surrounding and stabilizing the plaque. The plaque filled with lipids and necrotic debris grows slowly, initially it expands outwards with preserved lumen, but finally it causes narrowing of the vessel lumen, resulting in ischemia(95). The fibrous cap, initially stabilizing the plaque, can eventually become unstable. At this stage the lesion is labeled a "vulnerable plaque" because of the high risk of rupture. If rupture occurs, a thrombus is formed, which may lead to total occlusion and a life-threatening situation(96). Myocardial infarction, ischemic stroke, sudden cardiac death, chronic ischemic heart disease and peripheral arterial disease are among the most common manifestations of atherosclerosis.

Risk factors for atherosclerosis and CVD

The traditional CVD risk factors (age, male gender, smoking, family history, diabetes mellitus, hypertension) are nowadays well established. They are the result of extensive epidemiological studies, starting in the city of Framingham in the early 70s, there of the name "Framingham risk factors"(97). Some of them, as smoking, hyperlipidemia and hypertension, are modifiable, while others are not. The metabolic syndrome is a combination of metabolic CVD risk factors that often occur together, and consists of abdominal obesity, high triglycerides and total cholesterol, hypertension, and diabetes or insulin resistance (98). This cluster of risk factors is a large and growing problem in the industrial world. Although the traditional risk factors account for a substantial part of the CVD risk, their prevalence do not seem to fully explain for the cardiovascular diseases in the general population. Searching for other risk factors, so called non-traditional risk factors, has led to the observation of several new biomarkers of importance for CVD. Examples of these are C-reactive protein (CRP)(99, 100), fibrinogen(101), TNF α (102), cystatin C(100, 103, 104), IL-6(105), von Willebrand factor(vWf)(106), vascular cell adhesion molecule-1 (VCAM-1), inter cellular adhesion molecule-1 (ICAM-1), E- and P-selectin(107), antiphospholipid antibodies(108, 109) and antibodies to oxidized low density lipoprotein (anti-oxLDL)(110, 111). Many of these are markers of inflammation or endothelial activation/dysfunction, or immunological factors such as autoantibodies and cytokines, suggesting the

development of CVD to be a chronic inflammatory disease involving the immune system.

Atherosclerosis as a chronic inflammatory disease

During the last 20 years, atherosclerosis has become recognized as a chronic inflammatory disease(112, 113). The most accepted view is the "response to injury" hypothesis, where endothelial injury and the following responses are the initiating and driving forces behind the atheropathogenesis. Activation of the endothelium can be induced by risk factors for CVD, such as hyperlipidemia(114), hypertension(115) and hyperglycemia(116).

A major culprit in the initiation of the inflammatory process of atherosclerosis is low-density-lipoprotein (LDL). The formation of oxidized LDL(oxLDL) plays a significant role in the aethiopathogenesis of atherosclerosis(117). OxLDL is formed through oxidation of LDL trapped in the subendothelial space during early atherogenesis. Hypercholesterolemia leads to infiltration and retention of LDL in the arterial intima, initiating an inflammatory response in the arterial wall(118). Modification of LDL through oxidation leads to release of phospholipids that can activate endothelial cells(119). The activated endothelium expresses different types of leukocyte adhesion molecules, that causes circulating blood cells to adhere at the site of activation(120). VCAM-1 is upregulated in response to hypercholesterolemia, and the cells having counterreceptors for VCAM-1 (monocytes, lymphocytes), adhere to these sites (121). Chemokines produced in the intima stimulate the blood cells to migrate into the subendothelial space. In the intima, macrophage-colony stimulating factor induces monocytes to mature into macrophages. The maturation is associated with up-regulation of scavenger receptors and toll like receptors (TLRs). The scavenger receptors internalize a broad range of molecules and particles with pathogenic features, there among modified LDL(122). This process transforms the macrophage into a foam cell. The foam cell grow slowly, unable to degrade its content, ultimately leading to cell death and accumulation centrally in the plaque.

TLRs can, in contrast to scavenger receptors, initiate cell activation(123), and the activated macrophages produce inflammatory cytokines, proteases, and cytotoxic oxygen and nitrogen radical molecules. Dendritic cells (DCs) that patrol arteries may take up LDL components for subsequent antigen presentation in regional lymph nodes. In the normal artery wall, resident DCs are thought to promote tolerization to antigens by silencing T cells; however, danger signals generated during atherogenesis may activate DCs, leading to a switch from tolerance to the activation of adaptive immunity(124).

Endothelial activation also leads to rolling, adhesion and migration of T-cells into the site of inflammation. T cells are always present in the atherosclerotic lesion, predominately CD4+ cells. The atherosclerotic lesion contains cytokines that promote a Th1 response(125), leading to differentiaton of the T cells into Th1 effector cells, producing IFN- γ . IFN- γ is a proinflammatory cytokine that augments synthesis of TNF α and IL-1(126), further promoting atherosclerosis. T cell cytokines induce the production of many substances downstream in the inflammatory cascade, which can be detected in the peripheral circulation, e.g. CRP and IL-6. Subsequently, smooth muscle cells (SMCs) in the vessel wall are stimulated to migrate to and proliferate in the intima. Moreover, extra-cellular matrix production is stimulated, forming a fibrous cap around the cells(112, 127). This process ultimately leads to a thickening of the vessel wall and the formation of an atheroma. An advanced atherosclerotic lesion is characterized by a core of lipids and necrotic tissue covered by a fibrous cap, comprising SMCs and collagen(112). Activated macrophages and SMCs release matrix metalloproteinases

(MMPs), degrading the fibrous cap leading to subsequent rupture of the plaque and thrombus formation.

In addition to the proinflammatory process in atherosclerosis, partly due to Th1 cells and their cytokines, there are also protective mechanisms. Th2 cells, known to produce IL-10, have been associated with protection against atherosclerosis in murine models(128). In humans, increased levels of IL-10 have been linked to decreased risk of CVD(129). Regulatory T cells (T_{reg}), also producing IL-10, have been implicated in the protection against atherosclerosis. Mice transgenic for human apo B, immunized with apo B peptide vaccines can be protected from atherosclerosis even in the absence of an antibody response(130). The immunization is associated with an activation of T_{reg} , and administration of antibodies directed to T_{reg} blocks the atheroprotective effect of the vaccine(131). The importance of atheroprotective antibodies is discussed below.

Involvement of autoantibodies in the development of atherosclerosis

Except for being proinflammatory, oxLDL is also immunogenic, and the immune response against oxLDL is considered as a crucial event in atherogenesis(127). Neopeptides on LDL generated through LDL oxidation become targets for autoantibodies. Elevated levels of anti-oxLDL have been associated with progression of atherosclerosis (110, 132) and cardiovascular disease(110, 111). Although adaptive immunity is believed to have a net atherogenic effect, antiatherogenic effects of immune responses against oxLDL have also been described. Several experimental studies of rabbits and mice where oxLDL has been used for immunization demonstrated a positive correlation between high titers of anti-oxLDL and the degree of protection against atherosclerosis(133-135). The situation is more complex in humans, as often is the case. Different studies show positive or negative correlations, or no correlations, between anti-oxLDL titers and atherosclerosis or its features(135-139). Interestingly, titers of IgM and IgG antibodies to oxLDL have been found to show differences in their associations with coronary artery disease (CAD), suggesting that their biological roles differ(140-142). Thus, the repertoire of anti-oxLDL may include both "protective" and "pathogenic" autoantibodies. A possible mechanism for protective antibodies could be that under normal circumstances, anti-oxLDL may have a function in clearance of high levels of oxLDL. But, upon enhanced LDL oxidation (for example in cigarette smoking or inflammatory conditions), autoantibodies with a higher affinity, or targeting different epitopes on oxLDL, may be generated, aiding uptake by macrophages and thus enhancing foam cell formation and macrophage activation. Also, anti-oxLDL could enhance oxLDL uptake by macrophages through involvement of Fc receptors rather than scavenger receptors(143). The complexity of anti-oxLDL has been reviewed elsewhere(144). Finally, worth mentioning while discussing oxLDL/anti-oxLDL, is phosphorylcholine (PC) and its natural antibodies, suggested to possibly influence the development of atherosclerosis. PC is a part of oxLDL, more defined in its structure than oxLDL. Recent data suggest that anti-PC antibodies, especially of the IgM subclass, could have an atheroprotective effect, as low levels have been associated with increased risk of cardiovascular events(145, 146). The potential role of PC/anti-PC was recently reviewed by de Faire and Frostegård(147).

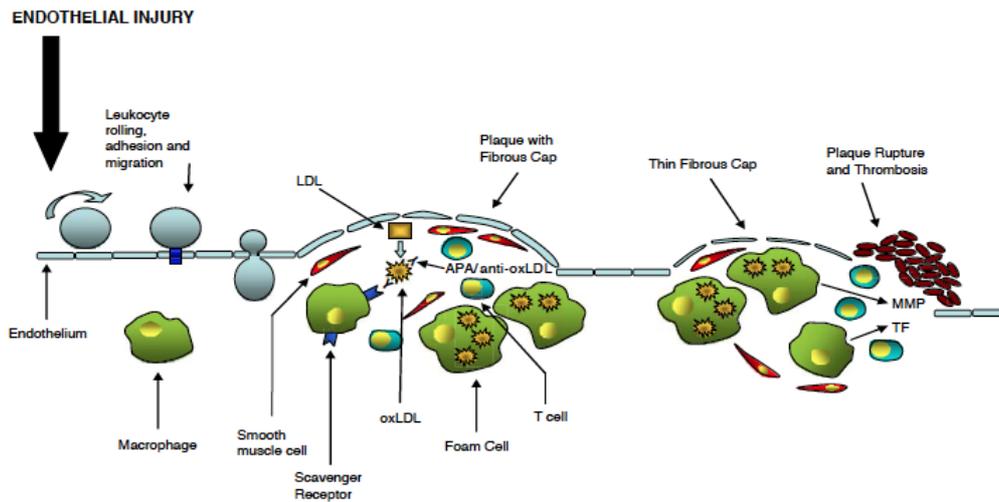


Figure 2. Inflammation in atherogenesis and atherosclerotic disease progression.
Adapted from Rhew and Ramsey-Goldman, with permission from the publisher.

Cardiovascular disease in SLE

CVD epidemiology

That SLE patients are prone to premature cardiovascular disease is nowadays an established phenomenon. In 1974, studies by Urowitz et al described the "bimodal pattern" in lupus, where a second mortality peak in the long term outcome of SLE patients related to cardiovascular disease was noted(27). In large series of SLE patients described since the 1970s, 6-75% of deaths were due to CVD (13, 24, 31, 33, 148, 149). Since the 70s, the cardiovascular research in SLE has been growing continuously, and the amount of data is now tremendous.

Clinical studies

The premature onset of CVD in lupus is well documented, with the age of first event noted on the average to be between 47-51 years(150-152). The overall prevalence of clinical cardiovascular disease varies between 6-10%(150-153). The risk of clinical cardiovascular disease in SLE patients varies between studies, but a 2 to 10 fold increase compared to the normal population has been reported(154-159). The relative risk is especially high among young patients(150, 160), although the absolute risk still is higher among older patients. In a classic study by Manzi et al, women with SLE in the 35-44 year age group were more than 50 times more likely to have an MI than women of similar age in the Framingham study(150).

Subclinical disease

Measurements of atherosclerosis are often referred to as subclinical CVD. Autopsy studies have demonstrated a prevalence of atherosclerosis in 41-53%(29, 148) of SLE patients, regardless of the actual cause of death. Carotid plaques and subclinical coronary artery disease have been demonstrated in up to 30-40% of lupus patients(161) (162-164) vs 9-16% of controls(161, 164). Most studies on atherosclerosis in lupus are cross sectional, but a handful of prospective studies have now also investigated progression of atherosclerosis(165, 166), (167-169). Progression also seems to be accelerated in SLE compared to control subjects(170).

While most studies have demonstrated that the development of atherosclerosis is accelerated in SLE, some groups have been unable to confirm these observations (Table 3).

Table 3. Selection of case-control studies on atherosclerosis prevalence in SLE							
	Surrogate measure of atherosclerosis	No of Patients		No of Controls		Plaques	Intima media thickness
Falaschi et al 2000(171)	Carotid ultrasound	26		26		Not measured	↑ among patients
Svenungsson et al 2001(172)	Carotid ultrasound	26 SLE cases	26 SLE controls	26 controls		↑ among SLE cases & controls vs controls	↑ among SLE cases vs SLE controls & controls
Asanuma et al 2003(164)	EBCT	65		69		↑ CAC among patients	
Roman et al 2003(161)	Carotid ultrasound	197		197		↑ among patients	↓ among patients
De Souza et al 2005(173)	Carotid ultrasound	82		62		↑ among patients	Not measured
Roman et al 2005(174)	Carotid ultrasound	101		105		↑ among patients	NS
Jimenez et al 2005(175)	Carotid ultrasound	70		40		↑ among patients	NS
De Leeuw et al 2006(176)	Carotid ultrasound	72		36		NS	↑ among patients
Lopez et al 2006(177)	Carotid ultrasound	30		27		↑ among patients	↑ among patients
Von Feldt et al 2006(178)	EBCT	152		142		↑ CAC among patients	
Chung et al 2006(179)	EBCT	93		65		↑ CAC among patients	
Kao et al 2008(180)	EBCT	157		157		↑ CAC among patients	Kao et al 2008
Rho et al 2008(181)	EBCT	109		78		↑ CAC among patients	Rho et al 2008
Colombo et al 2008(182)	Carotid ultrasound	80		80		↑ among patients	↑ among patients

Thomson et al 2008(170)	Carotid ultrasound	217	104	↑ among patients	NS
Huang et al 2009(167)	Carotid ultrasound	76	38	Not measured	↑ among patients
Reynolds et al 2010(183)	Carotid ultrasound	119	71	↑ among patients	↑ among patients
Gallelli et al 2010(184)	Carotid ultrasound	75	75	↑ among patients	NS
McMahon et al 2011(185)	Carotid ultrasound	250	122	NS	NS

EBCT: electron beam computed tomography

CAC: coronary artery calcium

NS: non significant

Methods to measure atherosclerosis

Intima media thickness (IMT) and plaques of extracranial carotid arteries, measured by B-mode ultrasound, is the most widely accepted non-invasive marker of subclinical atherosclerosis, which has been used in clinical and epidemiological studies to investigate the effects of established and non-traditional vascular risk factors(186). In addition, in view of its correlation with coronary atherosclerosis (187) and its capacity to predict incident coronary events (188, 189), carotid IMT has been proposed as a surrogate marker of coronary atherosclerosis(187, 190). Another non-invasive method to detect subclinical atherosclerosis is Electron beam computed tomography (EBCT), mostly measuring coronary artery calcium (CAC), also predictive of future cardiovascular events(191, 192).

Risk factors for atherosclerosis and CVD in SLE

Past studies have focused on evaluating risk factors for atherosclerosis, clinical events, or both. The results between studies are somewhat diverging, although some features are common for all. The traditional risk factors contribute, but cannot totally explain the increased risk. Instead, a combination of traditional and lupus-related factors contributes to the accelerated and premature atherothrombotic disease in lupus. The increased prevalence of atherosclerosis can explain part of the increased risk for CVD in SLE patients, but as SLE is a complex disease with the hallmark of immune dysregulation, inflammation, endothelial dysfunction and autoantibodies, CVD may also result from other mechanisms. Although carotid plaque and IMT are known to predict cardiovascular events in the general population(193), prospective studies on associations between subclinical atherosclerosis in SLE in relation to clinical events are still scarce.

Traditional risk factors

Some studies have investigated whether SLE patients have more traditional risk factors than controls. Patients with SLE have in some studies been more likely to have hypertension, diabetes mellitus, and lipid derangements(158, 194, 195). On the other hand, SLE patients have been found to have on average one less classic risk factor at time of their event compared to other populations with premature CVD(196). However,

after adjusting for traditional cardiovascular risk factors, patients with SLE still have an excess risk of CVD(155, 156).

Even though the traditional risk factors cannot fully explain the increased risk for atherosclerosis and CVD in SLE, their role cannot be ignored:

Age: Older age is naturally a strong risk factor for development of both atherosclerosis and clinical CVD(162, 197, 198) (164, 199). Many studies so far did not adjust for age when evaluating other variables. Given the strong influence by age and the fact that many potential CVD risk factors are influenced by age, such as blood pressure(200), inflammatory markers(201)and renal function(202), it is difficult to interpret the results of these studies.

Sex: Generally, male sex has been associated with increased risk of CVD (203, 204) and atherosclerosis(205, 206).

Smoking: Smoking is a risk factor for CVD(151), and was the only traditional risk factor that prospectively predicted cardiovascular events and mortality in two of our studies (paper I,II) and in a study by Toloza et al (198). Smoking is also associated with measures of atherosclerosis(205) (207, 208).

Hypertension: High blood pressure has been demonstrated to be associated with both increased risk of cardiovascular events(152, 204, 209, 210) and atherosclerosis(162, 206).

Hyperlipidemia: Hypercholesterolemia(150, 152, 211), high very low LDL (VLDL) and low high density lipoprotein (HDL)(172) are risk factors for clinical CVD. Hyperlipidemia is also associated with atherosclerosis(162).

Diabetes and overweight: Patients with SLE are more likely than controls to have the metabolic syndrome (212), which also is associated with subclinical atherosclerosis(199, 206, 213, 214). Overweight is both a risk factor for CAD(152) and atherosclerosis(199).

In prospective studies of atherosclerosis, mostly traditional risk factors(165, 166) and homocystein(168, 169), have been predictive of progression.

Lupus-related/non-traditional risk factors

Lipid dysregulation and LDL oxidation

The typical lipid profile in lupus is characterized by normal total cholesterol value, low or normal LDL, high VLDL and TG, and low HDL. This lipid profile is present in inactive untreated lupus and is more pronounced in active disease(195). Decreased activity of lipoprotein lipase (LPL)(215) or anti-LPL antibodies(216), and antibodies to HDL or apo-A1(217) are factors that might contribute to high TG and low HDL, respectively. Furthermore, abnormal lipid profiles in lupus have been associated with increased levels of TNF α (218), monocyte chemotactic protein 1 (MCP-1) and IL-6(219). The lipid profile in SLE is also affected by medications, such as steroid treatment and antimalarias. Steroids lead to an increase in total cholesterol, LDL and HDL, but also to some increase of TG levels(220). Antimalarias, on the other hand, have an atheroprotective effect on the lipid profile, by increasing HDL and decreasing total cholesterol and LDL(221). Renal disease including nephrotic syndrome gives elevation of cholesterol, LDL and TG levels(222).

Lipoprotein A (Lp(a)) is a lipoprotein subclass which comprises LDL-like particles with apolipoprotein a bound to apoB. High levels of Lp(a) is an independent predictor of CVD(223) and have also been reported to be elevated in SLE(172, 224, 225).

The importance of dysfunctional HDL has been the focus of several recent studies. The function of normal, anti-inflammatory HDL is to protect from atherosclerosis through transportation of cholesterol out of arterial walls and to prevent LDL from oxidation via anti-oxidant enzymes such as paroxonase1 (PON1)(226). In SLE, HDL can lose its anti-inflammatory properties, and instead becomes pro-inflammatory (pi-HDL). Pi-HDL occurs more frequently in SLE patients and is associated with plaque occurrence and coronary artery disease(227, 228). PON1 is an enzyme with antioxidant activity that circulates in plasma attached to HDL. Its physiological role is to prevent oxidation of LDL. Reduced PON-1 activity has been found in SLE compared to controls, and an association to CVD has also been suggested(229). Antibodies to HDL have furthermore been linked to reduced PON1 activity in lupus patients(230), suggesting that LDL oxidation could partly be due to the inhibitory effect from these antibodies on PON1(230).

Enhanced lipid oxidation has been suggested in lupus patients(231). Increased levels of anti-oxLDL have been found in up to 50% of SLE patients and have been associated with atherosclerosis and CVD(140, 172, 231-234). However, the role of anti-oxLDL in lupus related CVD is being debated, as some studies have not been able to confirm these associations(235-237).

Antiphospholipid antibodies

aPL are a heterogeneous group of antibodies, known to be highly thrombogenic. β_2 glycoprotein-1 (β_2 GP1) is nowadays considered to be the major target of these antibodies(238). Antiphospholipid syndrome (APS), which is characterized by aPL in combination with thrombotic/obstetric events, are discussed in a section below.

The association between aPL and thrombosis in SLE is a well established phenomenon (239-241). SLE patients with clinical CVD are more likely than those without to have positive lupus anticoagulant (LAC)(172, 210, 242). Prospective studies, thereamong our own study, have demonstrated an association between aPL and future cardiovascular events (159, 198, 243). While the link between aPL and clinical events is established, the association between aPL and atherosclerosis in SLE is debated. Some studies report associations between aPL and atherosclerosis(208, 244), whereas others have been unable to do so(161, 177, 242, 245).

The first evidence that aPL also could be involved in atherogenesis was provided by Vaarala et al, as they suggested that some aPL were able to crossreact with oxLDL in lupus(234), thus suggesting a possible link between thrombotic and atherosclerotic complications.

β_2 GP1 has been shown to bind oxLDL, and the colocalization of the oxLDL/ β_2 GP1 complex in atherosclerotic lesions implicate a role in the development of CVD(246, 247). One proposed physiological function of β_2 GP1 is to prevent the uptake of oxLDL via scavenger receptors. However, *in vitro* studies have shown that the simultaneous addition of a β_2 GP1 may enable the uptake of oxLDL/ β_2 GP1 by Fc-receptors on macrophages(248, 249). This could facilitate foam cell formation and represent an alternative pathway leading to atherosclerosis. Enhanced levels of oxLDL/ β_2 GP1 complexes and their respective antibodies have been demonstrated in SLE and in APS

patients in comparison to controls(177, 246, 250), and were furthermore associated with arterial thrombosis (246, 251).

Endothelial dysfunction and activation

Growing evidence suggests that endothelial dysfunction serves as a marker of inherent atherosclerotic risk. In 1977, Ross et al put forward the response to injury hypothesis, which proposes endothelial denudation as the initial stimulus for the development of atherosclerotic plaques(252). This hypothesis has evolved to emphasize endothelial dysfunction rather than denudation(112), and many risk factors for atherosclerosis such as elevated and modified LDL, smoking, hypertension and diabetes have been associated with endothelial dysfunction(253). Endothelial dysfunction transforms the internal layer of the artery from a non-adhesive, anticoagulant, impermeable barrier to one that supports leukocyte diapedesis, is procoagulant and the source of vasoactive molecules.

Endothelial dysfunction and/or damage can be evaluated in different ways. For example, it is possible to demonstrate dysfunction by measurements of products released from the endothelium such as nitric oxide, prostacyclin, von Willebrand factor (vWf) and adhesion molecules. Another method, more functional in its way of measuring endothelial reactivity, is flow-mediated dilation (FMD).

Studies investigating endothelial function through FMD suggest that endothelial dysfunction is present in lupus patients naive for vascular events(254-256) although the results are discordant(257). A metaanalysis evaluating the utility of FMD as a measurement of early vascular involvement was recently published by Mak et al. They concluded that endothelial-dependent FMD was impaired in lupus patients versus controls, whereas endothelial-independent FMD was not(258)

Elevated levels of adhesion molecules have been found in SLE patients, and correlate with disease activity(259). ICAM-1 are associated with increased coronary artery calcification(180, 181), as well as VCAM-1(181), the latter also demonstrated to be associated with SLE related CVD(257) and to predict arterial events in SLE patients(243). Other endothelial markers/adhesion molecules suggested to play a role in lupus related CVD are vascular endothelial growth factor (VEGF)(182), vWf(176, 243) and E-selectin(181, 183).

Another sign of endothelial dysregulation is that SLE patients are found to have high levels of circulating apoptotic endothelial cells (260). Endothelial apoptosis contributes to loss of endothelial integrity and thereby likely also to the initiation of atherosclerosis (261). Apoptotic endothelium is prothrombotic(262), partly because of increased expression of phosphatidylserine and loss of anticoagulant properties(263) and also due to activation of the TF cascade (260).

An imbalance between increased apoptosis and improper function of repairing mechanisms may aggravate vascular damage. Endothelial progenitor cells (EPC) are important contributors to endothelial repair, as they can proliferate and differentiate into mature endothelial cells. Decreased levels of EPCs have been implicated in increased CVD risk in SLE(264) as well as the general population(265). Type I IFN have been suggested to be able to deplete EPCs, supporting the role of IFN in the development of SLE related CVD(264).

Anti-endothelial antibodies (aECs) represent a heterogenous group of antibodies detectable in various autoimmune conditions(266). Little is still known about the antigens recognized by these antibodies. aECs are elevated in SLE, and have been suggested to be a disease activity marker in lupus(267). There may be a relationship between aPL and aECs, as it has been suggested that aECs may crossreact with oxLDL

and β_2 GP1 (268). Furthermore, the binding of aECs to endothelial cells has been demonstrated to involve β_2 GP1(269). aECs may also be able to induce endothelial apoptosis(270). Endothelial apoptosis can lead to the exposure of plasma membrane phosphatidylserine and subsequent binding of β_2 GP1(271). This could possibly explain some of the association between aECs and aPL, since aPL could be produced after the exposure of phosphatidylserine on ECs that are induced to undergo apoptosis by aECs(272).

Inflammatory markers

Levels of inflammatory markers such as TNF- α (218, 257), MCP-1 and IL-6(219), are increased in lupus patients with CVD. CRP is one of the most studied biomarkers with respect to cardiovascular risk, and has been demonstrated to predict cardiovascular events in the general population(99). In SLE, the results are diverging. In some studies, CRP was not associated with prevalent vascular disease(162, 199) nor did it predict atherosclerosis(233). However, other studies have suggested CRP to be a predictor of vascular events(197, 198) and to be associated with coronary artery calcification (CAC)(180). Barnes et al found an association between hsCRP and traditional risk factors in SLE and suggested that hsCRP could be of use in the assessment of cardiovascular risk, despite the fact that it is not valuable as marker of disease activity in SLE(273). Finally, hyperhomocysteinemia has been implicated as a risk factor of CVD and atherosclerosis in the general population(274), as well as in SLE(178, 199, 275).

Complement and CVD

End organ damage in lupus is associated with immune complex deposition and complement activation in target tissues. Complement activation may also be important for the development and progression of atherosclerotic plaques by stimulating endothelial cell activation and enhancing recruitment of leucocytes to inflammatory sites(276). Immune complexes in lupus can stimulate endothelial cells to express VCAM-1, which can promote recruitment of monocytes to the arterial wall(277). The anaphylatoxins and chemokines produced by complement activation may also promote inflammation and the atherosclerotic process. However, the role of the complement system in atherosclerosis development might be dual, as also protective mechanisms such as removal of apoptotic cells and cell debris from atherosclerotic plaques may be of importance(278).

C4d deposition on platelets is rather specific for SLE, and is associated with the presence of aPL(279). Sera from patients with aPL have the capacity to activate the classical pathway of complement, measured as deposition of C1q and/or C4d on platelets. The deposition has been associated with increased incidence of arterial thrombosis in SLE patients(280). The mechanism could be that aPL bind to platelets, creating immune complexes that are recognized by complement. This might lead to increased risk for thrombosis through platelet activation and complement-derived inflammatory mediators(280).

Other potential pathogenetic mechanisms involving complement are C2 deficiency(281) and variant alleles of MBL(282) both of which have been associated with CVD in SLE. Finally, elevated C3 levels, somewhat counterintuitive, have also been associated with CVD risk(170, 207).

Disease activity, damage and renal dysfunction

Disease duration and high SLICC damage-index scores have in many studies been demonstrated to be associated with premature CVD in lupus(150, 152, 161, 283).

Low glomerular filtration rate predicts arterial events in lupus(284). Faurschou et al recently demonstrated an disturbingly high cardiovascular risk among young patients with lupus nephritis, where the standardized ratio of observed to expected events was as high as 42(285). Renal disease and lupus nephritis have also been associated with accelerated atherosclerosis(170, 171, 205, 286) and serum creatinine has been identified as an independent predictor of IMT progression in SLE(170).

Cystatin C is a reversible inhibitor of cysteine proteinases that is produced by most cells. It can be used as a marker of glomerular filtration rate (GFR) because it is freely filtered at the glomerulus and reabsorbed and catabolized in the proximal renal tubules. It is not affected by gender and muscle mass, and it is more sensitive to small decreases in renal function. Cystatin C is believed to provide a more accurate measure of renal function than creatinine(287). The association between renal impairment and atherosclerosis and CVD is well established(288). In recent years, cystatin C has been reported as a prognostic marker of cardiovascular disease and death in the general population, even after adjustment for renal function measured by standard methods(104, 289, 290). Several large population studies have also demonstrated an association between inflammation and cystatin C(291-293). Thus, cystatin C may serve as an early marker for increased CVD risk resulting from subtle renal impairment, or perhaps could also participate directly in atherogenesis through its role in inflammatory processes(294).

Role of SLE treatment

Corticosteroids play opposing roles in CVD risk in lupus. Steroids may protect against CVD by reducing inflammation and disease activity, but the metabolic effects associated with their long-term use implicate increased risk. Accordingly, high doses of steroids have been shown to increase CVD risk in SLE(199, 210, 214, 220). On the other hand, Roman et al demonstrated that lower average prednisone dose and less aggressive immunosuppressive therapy such as cyclophosphamide was associated with atherosclerosis and carotid plaques in lupus patients(161, 168), indicating that aggressive control of disease activity may be important to reduce CVD risk. Also, in the context of SLE, steroids are a marker of the severity of the underlying inflammatory disease state. As such it is difficult to decide whether to attribute any possible association with the drug or the disease phenotype.

Hydroxychloroquine, a commonly used medication in the treatment of SLE, has been associated with lower burden of carotid plaques and with protection against CVD in lupus(161, 210, 220, 240, 244, 295, 296). The protective effect may be through antiinflammatory, antithrombotic(297) and lipid-lowering(298) properties.

Genetic predisposition

STAT4 was recently demonstrated to have a possible interference with cardiovascular disease in lupus, when we in paper III showed an association between a *STAT4* risk allele, stroke and aPL in SLE(299).

Another genetic finding is that CRP gene polymorphisms have an association with arterial vascular events in lupus(300).

MBL has also been implicated as a risk factor for CVD. Variant alleles of the gene exist, resulting in reduced levels and also a dysfunctional protein(301). Patients with low levels of functioning MBL are more prone to infections(301). Studies indicate an association between MBL deficiency and susceptibility for SLE(302), and low MBL concentrations have been related to the clinical course of SLE, e.g., complicating infections(303) and renal involvement(304). However, defects in MBL may also lead to increased risk for atherothrombosis. This association has been suggested in the general population(305), and in SLE(282, 306, 307), although the results have not always been consistent(308).

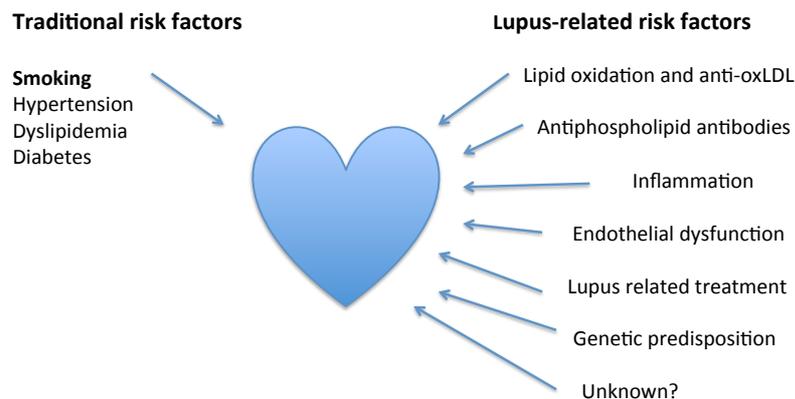


Figure 4. Premature cardiovascular disease in SLE

Antiphospholipid syndrome

The antiphospholipid syndrome (APS) is the most common cause of acquired hypercoagulability. APS is characterized by one or more thrombotic (venous or arterial) episodes or pregnancy morbidity in combination with the laboratory detection of antiphospholipid antibodies, aPL(309).

Ethiopathogenesis

The lupus anticoagulant (LAC) was a phenomenon discovered first in lupus patients, thereof the name. Later on, it was demonstrated that antibodies responsible for LAC activity *in vitro* recognize phospholipids(310). The name lupus anticoagulant is a misnomer, as LAC is not specific for lupus patients, and anticoagulant only *in vitro*. *In vivo*, a positive LAC test is strongly associated with enhanced risk of thrombosis.

The broad range of aPL is now known to include antibodies against anionic phospholipids (such as cardiolipin, phosphatidylethanolamine, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol), their putative protein cofactors such as β_2 GP1(238, 311), prothrombin, protein C, annexin V, or a complex between phospholipid and protein(312).

At present, the antibodies used in clinical practice for the diagnosis of APS are LAC, aCL IgG and IgM, and a β_2 GP1 IgG and IgM(309). The major antigenic target of this heterogeneous group of antibodies is the plasma protein β_2 GP1(238). LAC reactivity is thought to be mediated mainly by antibodies against β_2 GP1 and prothrombin(313, 314). The etiology and pathogenesis of APS is complex and multifactorial. Normal individuals occasionally have elevated levels of aPL(315), and as with other autoantibodies, the prevalence increase with age, especially among elderly patients with coexistent chronic diseases(316).

The development of APS is under the control of both genetic and environmental factors. Clinical manifestations of APS only occur in some patients with aPL(316). The fact that some patients have aPL for years and only develop clinical events under certain conditions has given rise to the theory that a "second hit" is sometimes required to trigger APS. Various factors might influence the development of events, such as specificity and titers of aPL, immobilisation, surgery, trauma, drugs and pregnancy(317). A well known environmental trigger for aPL are infections, both viral and bacterial. For example, Cytomegalovirus, Epstein-Barr virus, Parvovirus B19 and Mycoplasma pneumoniae have been associated with aPL, but also the clinical events(317). Supporting the influence by environmental factors on aPL production is the finding of aPL in spouses of SLE patients(318).

APS can be "primary", as the only feature, or "secondary", if associated with SLE or other non-lupus disease. Among SLE patients, 30-50% have aPL, and about a third of these develop secondary APS(316).

How these antibodies actually lead to thrombosis is still not known. A lead is that all of the tests used in clinical practice for diagnosing APS (aCL, LAC, a β_2 GP1) need β_2 GP1 for their optimal performance(309). Thus, β_2 GP1 has an important but unknown role in the pathogenesis of APS. The physiological function of β_2 GP1 is not totally known. It is thought that, after a conformational change of the protein (that occurs upon binding to anionic surfaces), new epitopes are exposed. The antibodies directed against the protein can then bind and stabilize it, inducing a new function(319).

Many theories have been proposed for how aPL induces thrombosis and foetal loss, but none of them have been proven with convincing evidence. Among the most popular hypotheses are activation of different cell types (endothelial cells, monocytes, platelets, neutrophils), inhibition of protein C, increased thrombin generation, complement disturbances and altered fibrinolysis(320). Ways by which aPL could promote development of atherosclerosis have been discussed earlier. For example, aCL can induce monocyte adherence to endothelial cells through upregulation of adhesion molecules(321), oxLDL/ β_2 GP1 complexes are localized in atherosclerotic plaques(246), and anti- β_2 GP1 may accelerate the uptake of oxLDL by macrophages(248).

Clinical manifestations

Venous thromboembolic events are the most common initial clinical findings in APS, and are generally also more common than arterial events(322). The site of the first event (arterial or venous) tend to predict the site of subsequent events(323), although some patients present cumulatively with both arterial and venous events.

As already mentioned, in SLE, the association between aPL and CVD has been demonstrated repeatedly. This is also true for the general population. Patients with coronary artery disease have higher levels of aPL than healthy controls(109) and aCL antibodies(324), particularly a β_2 GP1, are risk factors for myocardial infarction(108, 325-327). The association between aPL and CVD is especially strong with respect to

cerebrovascular disease, and the highest risk is associated with a positive LAC test (328, 329). LAC is furthermore established as the strongest risk factor among the aCL for thrombosis, irrespective of the site and type of thrombosis(330).

APS and atherosclerosis

The detection of atherosclerosis in primary APS has lagged behind that in SLE for the lack of suitable large study populations. There are however some studies that have focused on whether atherosclerosis is increased in this patient group. Although there is a theoretical background for the involvement of aPL in the pathophysiology of atherosclerosis, the results on atherosclerosis in APS through measuring carotid plaques or IMT are diverging.

Some studies demonstrated increased subclinical atherosclerosis in primary(331-336) or secondary (337) APS patients in comparison with controls, while others have not been able to confirm this association(175, 338).

Patients and Methods

Patients and study design (I-IV)

All patients at the Department of Rheumatology, Karolinska University Hospital Solna, who fulfilled four or more of the 1982 revised American College of Rheumatology Criteria for classification of SLE(6) were asked to participate. 208 agreed and were included in the cohort during the period July 1995-December 1999. The initial investigation included an interview and a physical examination by a rheumatologist and blood samples were collected after overnight fasting. This cohort is the basis for paper I and II. In paper I, 182 of the 208 patients were free of previous arterial events and were included. Mean time of followup was 8.3 ± 1.2 years in paper I, and 12.3 ± 3.3 years in paper II.

In 2004, reinclusion of patients began, and continued until 2010. Surviving patients from the original cohort, as well as new patients diagnosed since 1999, were asked to participate. This cohort is the basis for paper III and IV. In paper III, in addition to patients from the Karolinska cohort (N=293), patients from Uppsala(N=131), and Lund (N=154) were included. If patients were related only the first case in each family was included; thus the study population included unrelated patients. Matched controls were genotyped to give a comparison to the local genetic background (N=651). In paper IV, 367 patients were included. Paper I and II have a prospective design, whereas III and IV are cross-sectional.

The studies were approved by the local Ethics Committee of the Karolinska Hospital, Stockholm, Sweden. All subjects gave their informed consent before entering the studies.

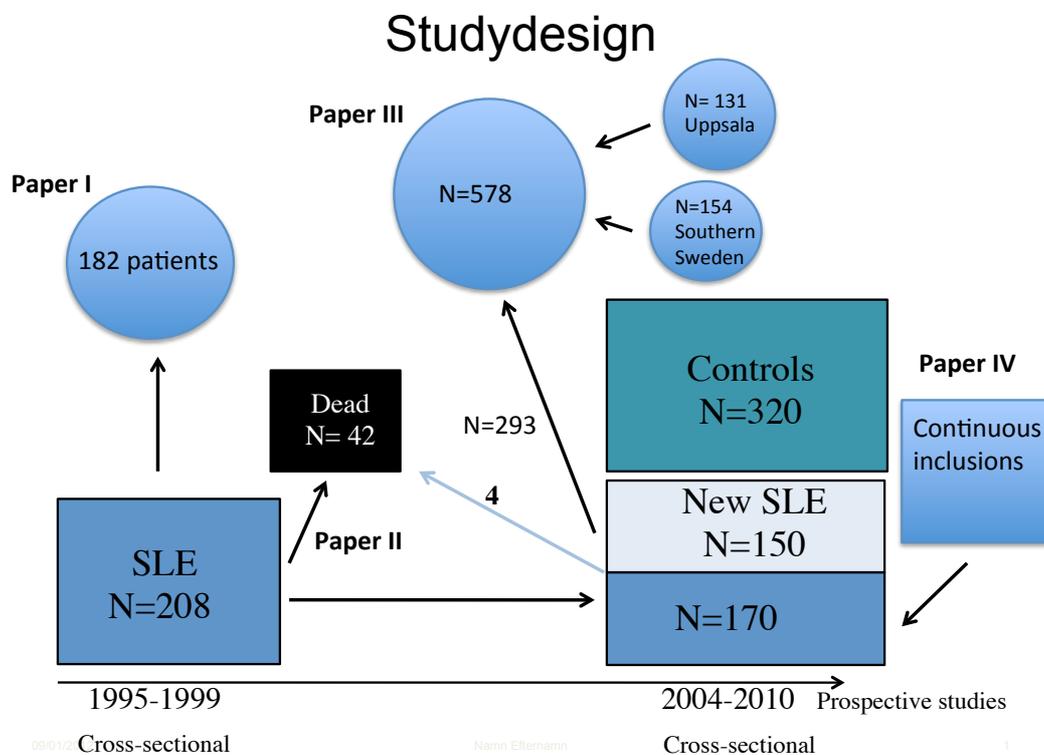


Figure 4. Studydesign

Disease activity and damage (I, II, IV)

SLE disease activity was determined with SLAM(9) and organ damage with the SLICC/ACR damage index(153). A SLAM score>6 was considered active disease(339). These were done at the initial inclusion for all patients.

Definition of vascular events (I,III,IV) and determination of causes of mortality(II)

Vascular events were defined as:

- 1) Ischaemic heart disease (IHD): myocardial infarction (MI, confirmed by electrocardiography and a rise in plasma creatine kinase, muscle and brain fraction (CKMB) or troponin T) or angina pectoris (confirmed by exercise stress test).
- 2) Ischaemic cerebrovascular disease (ICVD): cerebral infarction (confirmed by computer tomography) or transitory ischemic attack (TIA, defined as transient focal symptoms from the brain or retina with a maximum duration of 24 hours).
- 3) Ischaemic peripheral vascular disease (IPVD): intermittent claudication or peripheral arterial thrombosis/embolus (confirmed by angiogram or Doppler flow studies).
- 4) Death due to ischaemic vascular disease: death due to myocardial infarction, heart failure, sudden death, cerebral infarction, generalized atherosclerosis as stated by death certificate.
- 5) Venous thromboembolism (VTE): defined as deep vein thrombosis, confirmed by venography or ultrasonography and/or pulmonary embolism, confirmed by radionuclide lung scanning or angiogram.

Any arterial event (in paper III, IV)= any of 1-3

1-3,5: used in paper III, IV

1-4: used in paper I

4: used in paper II

In paper II, survival status was followed up in the national population registries through March 26, 2010. Death certificates were collected from the Cause of Death Register of The National Board of Health and Welfare. When available, autopsy protocols were collected from the Department of Pathology Karolinska University Hospital, and from the Department of Forensic Medicine.

Genotyping (III)

The SNP rs10181656 had previously been genotyped using the GoldenGate assay (Illumina inc.) and the SNPstream system (Beckman-Coulter inc.) in two Swedish SLE cohorts from Mid and Southern Sweden. These two cohorts comprised 424 and 154 patients respectively. From Mid Sweden 457 controls were included, and from Southern Sweden 194 controls were available.

Laboratory measures (I, II)

High sensitivity C-reactive protein (CRP), α -1 antitrypsin, fibrinogen and Serum Amyloid A (SAA) were measured using BN ProSpec System (Dade Behring). C3 and C4 were analyzed using IMMAGETM and C3d using an ARRAYTM system (both instruments from Beckman Coulter). Albumin, apolipoprotein A1, apolipoprotein B, homocysteine and Cystatin C were measured on an Architect Ci8200 analyzer (Abbott Laboratories). Creatinine, HDL-, LDL-and total cholesterol, triglycerides and urea were analyzed on LX20 chemistry analyzer (Beckman Coulter).

Enzyme-linked immunosorbent assays (ELISA) were used to measure soluble Vascular cell adhesion molecule (sVCAM-1), von Willbrand factor (vWF, antisera from Dako)

calibrated against Liatest (Diagnostica Stago) and IL-6 (high sensitivity ELISA, R+D systems). Intra-assay coefficient of variations for the ELISAs were <7%. Plasma electrophoresis was analyzed on agarose gels.

Autoantibodies(I-IV)

In paper I and II, ANA were analyzed by immunofluorescence on HEp-2 cells (Immunoconcepts, Sacramento, CA, USA) and auto-antibodies to SSA, SSB, Sm and RNP using ANA-profile ELISA (PharmaciaDiagnostics), Innolia Immunoblot (Innogenetics), and Immunodiffusion (Immunoconcepts). Anti-doublestranded DNA (dsDNA) antibodies were determined by Crithidia lucillae kinetoplast assay (Immunoconcepts). Anticardiolipin antibodies (aCL) were measured by ELISA using ethanol fixed cardiolipin (Sigma-Aldrich) and HRP-conjugated rabbit anti-human IgG and IgM (Dako). Positivity was calibrated against Harris standard (Louisville). a β ₂GP1 IgG were analyzed by ELISA (Orgentec).

In paper III and IV, aCLIgG/IgM and a β ₂GP1 IgG were analyzed by ELISA (Orgentec, Mainz, Germany). Anti-prothrombin (aPT, IgG) were analyzed by ELISA (Orgentec)(paper II). Lupus anticoagulant (LAC) was determined using a modified Dilute Russel Viper Venom method, (Biopool) using Bioclot LAC (I-IV).

Statistics(I-IV)

Characteristics of the study populations were described first using descriptive statistics such as medians and interquartile range or mean \pm SD for continuous variables, and as percentages for categorical variables. For continuous variables, comparisons were made by ANOVA/t-tests, Mann-Whitney U-test or Kruskal-Wallis test as needed. Chi-square and Fischer's exact test were used to evaluate categorical variables as appropriate.

Papers I and II: Univariate and multivariable-adjusted Cox proportional hazards regression models were used to evaluate a number of baseline variables as risk factors for the first CVE (I) and mortality (II). Because of limited number of first CVEs and deaths, we restricted the amount of variables in the multivariable models to four.

In paper I, baseline variables were sorted into functional groups. After adjusting for age, the variables from each group that were representative and most significantly associated (i.e. had the smallest p-value) with future CVEs were included in a multivariable-adjusted model.

In paper II, the two predictors most strongly associated with mortality after age-adjustment (in terms of p-value) were retained in all multivariable analyses. Thereafter each baseline variable was considered separately, i.e. included one by one in a multivariable model containing age and the two other predictors. Furthermore, models were stratified by sex, steroid treatment, and nephritis to investigate possible effect modification by these factors. The SMR was calculated using age-, sex- and calendar year specific mortality rates for the Swedish population. SCORE was calculated using Swedish heart score (www.heartscore.org).

Paper III: Fischer's exact test was used to test for Hardy-Weinberg equilibrium of genotypes. OR and 95% CI were calculated from 2x2 contingency tables and Mantel-Haenszel estimates were used for summary measures of the two investigated patient groups from mid and southern Sweden. Univariate and multivariable-adjusted logistic regression analyses estimated the impact of risk factors on ICVD and IHD events.

Paper IV: Multivariable-adjusted logistic regression models estimated the association between smoking and aPL status. Smoking was defined as never, former, current and ever (=former+current). Never smoking was used as reference in all calculations. Stratifications for various variables (sex, warfarin treatment, previous arterial/venous events, and by exclusion of patients who stopped smoking after event) were made to evaluate effect modification and possible reverse causation.

All statistical analyzes were performed using JMP software (SAS institute, North Carolina, USA), with the exception of SMR, where SAS 9.2 was used. A p-value ≤ 0.05 was considered statistically significant.

Results

Paper I

Predictors for the first cardiovascular event in patients with systemic lupus erythematosus – a prospective cohort study

Patients with SLE have increased risk for cardiovascular disease. Few studies have evaluated risk factors for cardiovascular events (CVE) prospectively. Therefore, we investigated traditional and lupus-associated risk factors for the first ever CVE longitudinally in a cohort of 208 patients. 26 patients had a previous event and were excluded.

Results

182 patients were included and followed up after an average of 8 years during which time 13% (24 patients) had a first CVE. When evaluating the traditional risk factors in age-adjusted Cox regressions only current smoking was associated with CVE. sVCAM-1, vWf, low plasma albumin, fibrinogen and aPL, predicted CVEs. aSSB was inversely associated with CVE. Of SLE manifestations, arthritis, pleuritis, previous venous occlusion and absence of thrombocytopenia predicted events. In a multivariable Cox regression model restricted to four variables age, any positive aPL, vWf and absence of thrombocytopenia remained as predictors for the first CVE.

Conclusions

We identified cigarette smoking as the most important traditional CV risk factor, and demonstrated that aPL, high levels of endothelial markers (sVCAM-1 and vWf) and absence of thrombocytopenia were associated with the first CVE. This indicates that endothelial activation and damage, as well as coagulation dysregulation are important for CVD in lupus patients. Furthermore, the risk of CVEs seems to differ between subgroups of patients, where patients with manifestations such as arthritis and serositis, often associated with more systemic inflammation, may be at a higher risk than patients with more mild disease represented by for example photosensitivity and aSSA/SSB.

Paper II

Risk factors for cardiovascular mortality in patients with systemic lupus erythematosus- a prospective cohort study

The prognosis for patients with SLE has improved over the years. As patients with SLE live longer, cardiovascular disease has become more evident as a cause of morbidity and mortality. While death due to infections and active disease has decreased, CVM shows no such decline. There are a lot of studies examining risk factors for overall mortality in SLE, but few have specifically investigated risk factors for CVM in lupus. In this study, we prospectively examined a set of potential risk factors and the European heart SCORE in a SLE cohort of 208 patients. We determined causes of death and investigated baseline markers for all-cause mortality and specifically for CVM.

Results

After a mean follow-up time of 12 years, 42 of 208 patients died. The SMR was 2.4. Almost 50% of deaths were classified as CVM. SCORE underestimated CVM in this population, but not significantly. Several baseline parameters differed between deceased and surviving patients and persisted after adjustment for age. Age, high levels of cystatin C and established arterial disease were the strongest risk factors for all-cause mortality and CVM. In multivariable-adjusted Cox regression, after adjusting for these variables, only smoking from the traditional risk factors predicted CVM. sVCAM-1, hsCRP and warfarin treatment also remained significant. Stratification for sex, steroid treatment and nephritis did not change the results.

Conclusion

Cardiovascular disease was the main cause of mortality in this study. Age and established arterial disease were, as expected, strong risk factors for mortality. Except for smoking, traditional risk factors did not predict CVM. Instead, high levels of cystatin C, inflammatory and endothelial markers differ between patients with a more favourable vs. poor prognosis.

Paper III

A STAT4 risk allele is associated with ischaemic stroke and phospholipid antibodies in systemic lupus erythematosus

While overall prognosis in SLE has improved, vascular mortality seems to remain constant. Nontraditional risk factors such as inflammatory markers and antiphospholipid antibodies contribute to this increased risk. Also, genetic predisposition has been demonstrated to be associated with risk of CVD. After the MHC region, STAT4 is one of the strongest susceptibility factors in SLE. We investigated a possible association between the previously replicated SLE risk genotype in STAT4 (SNP rs10181656) and vascular manifestations and aPL in SLE.

Results

578 patients and 651 controls were available for analysis. The *STAT4* risk allele was more common among SLE patients with a history of arterial events, more specifically those who had experienced ICVD. There was a dose-dependent relationship between previous ICVD and number of risk alleles, where homozygous patients had the highest for ICVD. *STAT4* was also associated with occurrence of more than two aPL, and there was a dose-dependent relationship between the number of *STAT4* alleles and percentage

of patients with ≥ 2 aPL. Adjustment for available known risk factors did not attenuate the association between STAT4 and ICVD. Positive aCL IgG also remained as an independent risk factor for ICVD.

Conclusions

Patients with the *STAT4* risk allele have increased risk of ICVD. *STAT4* is also associated with prothrombotic aPL. Genetic predisposition is an important risk factor for ICVD in SLE patients, and aPL may be one underlying mechanism.

Paper IV

Cigarette smoking and antiphospholipid antibodies in systemic lupus erythematosus

Smoking is a known environmental risk factor for development of autoimmune disease, for example in RA and SLE. In RA it has been suggested that patients with a certain genetic predisposition (the "shared epitope") have a higher risk of developing RA, and more specifically, ACPA-positive RA. In SLE, smoking has been associated with the presence of anti-dsDNA, suggesting that smoking may trigger autoantibodies. aPL are often present in SLE patients, and is an established risk factor for thrombosis. As smoking is known to induce autoantibodies, and both smoking and aPL are associated with cardiovascular disease, we investigated a possible association between the two in a cohort of 367 lupus patients.

Results

aPL seropositivity varied with smoking status. When comparing ever vs. never smokers, ever smokers were more likely to be positive for aCL IgG, a β_2 GP1 and LAC. When splitting ever smokers into former and current, former smokers were most likely to be aPL positive, followed by current and never smokers. In multivariable analyses, adjusting for age, sex, and age at diagnosis, the association between former smoking and occurrence of aCL IgG, a β_2 GP1 and LAC remained. Sex stratification did not change the results. In the warfarin free patients former smoking and aPL were no longer significantly associated. Also, in the stratum of patients who did not have history of events, there was no association between smoking and aPL. However, when excluding patients who stopped smoking after event, the association remained.

Conclusion

Smoking was associated with the occurrence of aPL. Specifically, former smoking had the strongest impact on aPL positivity. The possibility of reverse causation (i.e. that aPL positive smokers who experienced an event were more likely to stop smoking, and therefore be classified as former smokers, thus the association) cannot be definitely excluded. It will be investigated in forthcoming studies.

General Discussion and future perspectives

This thesis has focused on investigating risk factors for cardiovascular disease in SLE, the main cause of long-term morbidity and mortality among these patients. Traditional risk factors, lupus related risk factors, systemic inflammation, endothelial dysfunction, autoantibodies and genetic aspects have been in focus. The findings are very interesting and shed light on some potential pathogenic mechanisms for cardiovascular disease in SLE.

Causes of death

In paper II, we investigate causes of death and risk factors especially for cardiovascular mortality in lupus. The estimated SMR of 2.4 is consistent with other recent studies(24), as well as the observed survival rate of 80% after 12 years(13, 32, 340). In our study, cardiovascular death accounted for nearly 50% of deaths, the percentage in other studies vary but generally, CVM account for a substantial part in most recent studies(13, 24, 31). We used the same strategy as most previous studies to determine causes of death, i.e. we relied predominately on death certificates, autopsy reports and medical charts(31). Some studies also used information from the family(17) and from discussions with the patients doctor(25, 33). Two other large studies on the other hand(23, 24), one of which is a multicenter study(24), used ICD-codes (international classification of diseases) to determine causes of death, the same method as the Cause of Death Registers use. National mortality data is based only on the ICD-codes derived from the death certificate, and refers to the specified underlying cause of death. As we used additional information to determine cause of death, the results may differ. It is therefore also difficult to compare the results from different studies and it is not possible to calculate cause-specific SMRs. Generally, the results from studies on mortality in lupus do not always support each other in respect to causes of death and risk factors. The differences may result from the above described methodological variations, patient selection and ethnicity, follow-up, definition of study variables, statistical analyzes and other confounding factors.

Cystatin C

Cystatin C emerged as a novel and strong predictor for CVM and all cause mortality paper II. A plausible link between increased cystatin C and impaired cardiovascular outcome is reduced renal function. However, in our study, other measures of renal function did not predict cardiovascular mortality, and other studies have also demonstrated that cystatin C is an independent predictor of poor prognosis, regardless of renal function(290, 341). It is possible though, that the prognostic value of cystatin C is due to its ability to detect preclinical renal dysfunction, not caught by ordinary measurements of GFR. In most studies, creatinine based formulas were used, not direct measurements of GFR, hence, it's difficult to determine the extent to which cystatin C concentration reflects kidney function. Inflammation, associated with atherogenic changes, may be another mechanism linking cystatin C to cardiovascular risk. High cystatin C levels correlated with high levels of CRP(291). Although it is possible that this association also could be the result of the presence of renal dysfunction(291, 292, 342), it has been suggested that high cystatin C levels are directly related to both inflammation and atherosclerosis(343). The cystatin C gene is one of the so-called "house-keeping genes". Cystatin C is an inhibitor of lysosomal and cysteine proteinases and it is produced in almost all nucleated cells at a stable rate. There is evidence that an imbalance between elastolytic enzymes and their inhibitors, cystatin C being one, are involved in the pathogenesis of atherosclerosis(344, 345). The increased levels of

cystatin C may reflect an attempt to counterbalance a potentially damaging increased elastocytic activity stimulated by inflammatory cytokines, the latter also associated with atherosclerosis(344). A recent study by Lertnawapan et al found that cystatin C levels (but not other measures of renal function) were higher in lupus patients than controls, and that they were associated with systemic inflammation, but not with atherosclerosis(346). Also in RA, levels of cystatin C have been shown to correlate with disease activity, in contrast to other measures of renal function(347). Further research is required to gain insight into the true significance of increased cystatin C concentrations in these various clinical settings.

Inflammation and endothelial activation

In paper I and II, the importance of inflammatory markers as predictors for cardiovascular outcome, such as fibrinogen and hsCRP, was demonstrated. Inflammatory markers have repeatedly been shown to be important risk factors for cardiovascular morbidity and mortality in the general population(99, 348, 349). Also in SLE, the relationship between inflammatory markers and cardiovascular outcomes have been investigated, although prospective studies are more scarce(172, 180, 198) but the impact on mortality has not previously been well studied.

What was seen for inflammatory markers can also be applied for markers of endothelial activation/dysfunction. sVCAM-1 was in papers I and II, and vWf in paper I, strongly associated with cardiovascular outcomes. When the endothelium is activated, as in inflammatory conditions, these biomarkers are upregulated. sVCAM-1 is an adhesion molecule, enhancing adhesion and rolling of leukocytes and subsequent migration into the subendothelial space, thereby enhancing the inflammatory and atherosclerotic process(350). It has been shown that sVCAM-1 is associated with increased risk for clinical CVD and atherosclerosis in the general population(351). In lupus, sVCAM-1 has been linked to active disease(259), nephritis(352), atherosclerosis(176, 181) and manifest CVD(257). However, in the context of SLE mortality, endothelial markers have not previously been investigated. Increased plasma levels of vWf are present in acute coronary syndromes and has pro-thrombotic effects as it promotes aggregation of platelets(106). In lupus, high levels of vWf could be attributable to pro-inflammatory cytokines and autoantibodies, both demonstrated to be able to cause release of vWf from endothelial cells(106, 353). Previous studies have suggested the importance of endothelial activation and dysfunction in lupus related cardiovascular disease, and we extend these findings by showing that increased levels of endothelial markers are strong predictors of both cardiovascular events and mortality

Antiphospholipid antibodies

Another important risk factor for the development of thrombosis are aPL. In papers I, III and IV, the significance of these autoantibodies in cardiovascular morbidity and potential pathogenesis is suggested. In paper I, aPL predicted the first arterial event, and in paper III aPL were associated with ICVD. aPL are well established risk factors for cardiovascular morbidity, both in the general population and in lupus(240, 241, 325, 326, 329). However, in paper IV, aPL did not influence the risk of CVM. This might be explained by the fact that aPL seem primarily to be a risk factor for ICVD rather than IHD(299, 322, 328, 329), and only one patient died from ICVD. In paper III, the *STAT4* risk allele was associated with aPL, thereby suggesting a possible pathogenic mechanism by which the susceptibility gene may predispose for ischemic events. In agreement with these results, *STAT4* has also been shown to be associated with APS(354). However, other studies examining the link between *STAT4* and CVD in RA

and patients with chronic kidney disease did not find any apparent associations(355, 356). Other mechanisms by which STAT4 could promote cardiovascular events are through inducing a pro-inflammatory behavior in endothelial cells(357) or proliferation of vascular smooth muscle cells(358). Promotion of differentiation of T helper cells into Th1 cells could be another mechanism, believed to be of importance for the development of atherosclerosis, as previously discussed(124).

Smoking

The importance of smoking, one of the most established traditional risk factors for CVD, was demonstrated in papers I, II and IV. In papers I and II, smoking was the only traditional risk factor that predicted cardiovascular outcome in multivariable-adjusted analyzes. The importance of smoking for the development of CVD is established and needs no further description. The relative contribution of smoking to the development of cardiovascular disease in lupus differs between studies, but some have found an association both with subclinical atherosclerosis and clinical CVD(198, 207). Smoking has detrimental effects on the immune system(359), and it has been shown to increase the risk for both RA and SLE(80, 360). In RA, smoking is important for the development of ACPA positivity, especially in patients with the genetic predisposition referred to as the "shared epitope"(360). In lupus, smoking has been shown to be associated with anti-dsDNA, suggesting pathogenic mechanisms (85). In paper IV, a strong association between former smoking and aPL was found. The reason for this association could be that smoking induces the production of aPL, as was also suggested for anti-dsDNA. The pathogenic mechanisms behind such an induction remains to be determined. Another plausible explanation for the observed between former smoking and aPL could be "reverse causation", described in the discussion in paper IV. Nevertheless, our observation is important. Patients who had a history of smoking and had aPL were at much greater risk of having a history of venous/arterial events compared to those who had either risk factor (ongoing analyses). Thus smoking may be considered as another type of "second hit" leading to thrombotic events in aPL positive patients. Infections, trauma, pregnancy has previously been suggested to constitute such "second hits" in patients with aPL/APS(317) and in the catastrophic APS(361). Taken together, our results demonstrate that aPL are very important risk factors for cardiovascular morbidity in lupus, that genetic factors influence the occurrence of aPL, and that smoking history is associated with these autoantibodies.

Disease activity and lupus phenotype

SLE patients with more severe disease, represented by high scores on activity- and damage indices clearly have worse prognosis(20, 362, 363). Patients with higher disease activity and damage also have higher risk of cardiovascular disease(241, 364, 365). As cardiovascular disease is included as an item in the SLICC damage index, it can sometimes be difficult to determine cause and effect determined by this damage index.

The finding in paper I that absence of thrombocytopenia was a risk factor for the first CVE was unexpected and not consistent with previous studies. Thrombocytopenia, a usual finding SLE patients, is usually associated with active disease(366, 367), and has been associated with mortality in several studies(366, 367). Why we found an inverse association is difficult to explain. Theoretically, thrombocytopenia may, as a "natural anticoagulant", protect against thrombotic events, but this hypothesis has to be tested in larger samples.

In paper I, patients with arthritis and serositis, often representing patients with more systemic inflammation, had a higher risk of CVE. In both paper I and II, aSSA and/or aSSB positivity was inversely associated with cardiovascular outcomes. In paper II, anti-dsDNA and neurological manifestations were risk factors for mortality. In clinical practice, patients who have aSSA/-SSB often have a milder clinical course with musculoskeletal problems and skin manifestations, while anti-dsDNA positive patients more often have tendency to develop nephritis, serositis and other more serious manifestations. Our observations are in agreement with others, where aSSA and photosensitivity have been inversely linked to mortality (19, 368), while renal impairment as measured by estimated GFR, was a risk factor for CVD(369). Thus differences in autoantibody profile and clinical course represent different lupus phenotypes, and are likely partly due to genetic differences. Further studies are ongoing to delineate the different SLE phenotypes.

Thoughts for consideration

Many studies use the terms “atherosclerosis” and “cardiovascular disease” as if they were interchangeable. Of course, atherosclerosis is a risk factor for the development of cardiovascular disease (i.e. clinical events; coronary artery disease, ischaemic cerebrovascular disease and peripheral vascular disease), at least in the general population, but it should not be forgotten that there are individuals with advanced atherosclerosis who never develop CVE. Prospective studies on the association between atherosclerosis and clinical CVD in lupus are still very scarce. To our knowledge, there has only been one abstract on this topic, still not published, presented at ACR in 2008(370).

Furthermore, studies investigating risk factors for CVD vary with respect to definition of outcome. Some studies only focus on coronary artery disease, others on the combination of IHD, ICVD and IPVD, and still others combine arterial and venous events. It is therefore difficult to compare studies, as risk factors for the different vascular manifestations may be different.

Finally, vascular events may result from several pathophysiologic mechanisms; some may be primarily thrombotic, others may be the result of atherosclerosis, and still others may be the result of an ongoing inflammatory process (vasculitis). However, in many instances, atherosclerosis and thrombosis are likely to occur together, and it is often difficult to exactly determine the underlying pathophysiological mechanisms.

Future perspectives

From the SLE cohort included 2004 and forward (“SLE 2004+”), we have data on 281 SLE patients and 281 controls, matched for age, sex and region of living. Carotid ultrasound has been performed in all patients and controls. We have preliminarily analyzed these data, and surprisingly, only IMT, but not the amount of plaques, differed between SLE patients and controls (crude data, ongoing analyses). We are now going to analyze these data more carefully in subgroups of patients and controls and investigate risk factors for atherosclerosis. Prospectively, it will be interesting to do a follow-up of these patients to evaluate the prognostic value of baseline IMT/plaques for the development of clinical CVD.

An example of an interesting biomarker worth further investigation is cystatin C both in relation to atherosclerosis and future events. A good approach would be to measure kidney function with for example iohexol clearance, to be able to evaluate the importance of cystatin C as a prognostic marker beyond that of renal function (as

discussed above). Unfortunately, we do not have these kinds of measurements in our cohort, but it could be something to consider for the future.

Future studies will also focus on looking into the temporal relationship between smoking and aPL, trying to evaluate the possibility of reverse causation. Also, we have not investigated whether this relationship is influenced by genetic predisposition, in a similar way as the occurrence of autoantibodies in RA. Finally, animal studies in a similar design as used by Rubin et al(86), could be considered to evaluate the association between this environmental factor and aPL.

Osteoporosis is a common complication in patients with SLE. In all patients mentioned above, “SLE 2004+”, Dual-energy X-ray Absorptometry (DXA) has been performed, and we also have measurements on body composition. A lot of information is gathered in these data, which have not yet been analyzed. As osteoporosis has been demonstrated to be associated with atherosclerosis, common underlying mechanisms are plausible and will be investigated in forthcoming studies. We plan to have cooperation with endocrinologists to be able to utilize the information in the best way possible.

Another project is focusing on circulating microparticles, which to some extent can be regarded as surrogate measures of cell populations, which are either activated or subject to enhanced apoptosis.

Other ongoing studies are those focusing on the genetic aspects of SLE, where we will continue to work together with geneticists in large international collaborations.

All taken together, future studies will have different angles of approach further trying to elucidate the enigma of the increased risk for CVD in SLE patients.

Conclusions

In this thesis I aimed to discuss the pathogenesis and risk factors for cardiovascular disease (CVD) and mortality in Systemic Lupus Erythematosus (SLE), and I conclude the following:

- Patients with SLE have a higher mortality rate compared to the normal population (SMR 2.4) and CVD is a predominant cause of death
- Except for smoking, traditional risk factors seem to have low impact on the cardiovascular mortality (CVM) in SLE. Instead, other predictors such as cystatin C, inflammation and endothelial activation seem to predict poor outcome.
- In a similar way as for CVM, risk factors for the first cardiovascular event (CVE) are represented by smoking and activation of the endothelium, and by the presence of antiphospholipid antibodies (aPL).
- Sub-phenotypes of SLE seem to have differentiated risk profiles for CVD and mortality, where patients with aSSA/SSB antibodies and/or skin manifestations have a better prognosis than patients with more severe organ manifestations.
- Genetic predisposition, represented by a *STAT4* risk allele, increases the risk of ICVD in SLE patients, where aPL may be one underlying mechanism.
- Smoking is associated with the occurrence of aPL. Whether this is due to the ability of smoking to induce these autoantibodies remains to be determined.
- Taken together, genetic predisposition in combination with smoking, inflammation, endothelial activation and aPL are major risk factors for CVD and mortality in SLE, which may act separately, or in combination by common pathophysiological mechanisms, to increase the burden of CVD in SLE.

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