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NOVEL IMMUNOLOGICAL MECHANISMS AND FACTORS IN SYSTEMIC LUPUS ERYTHEMATOSUS-RELATED CARDIOVASCULAR DISEASE

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The capacity to learn is a gift
The ability to learn is a skill
The willingness to learn is a choice

Frank Herbert

Sir,
In my heart there was a kind of fighting that would not let me sleep…

We know what we are but know not what we may be…

William Shakespeare

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. **Cederholm A,** Svenungsson E, Jensen-Urstad K, Trollmo C, Ulfgren A-K, Swedenborg J, Fei G-Z and Frostegård J. Decreased binding of Annexin V to endothelial cells- a potential mechanism in atherothrombosis in patients with systemic lupus erythematosus. Arterioscler Thromb Vasc Biol. 2005 Jan; 25(1): 198-203

II. **Cederholm A**, Su J, von Landenberg P. and Frostegård J. Effects of IVIG and anticardiolipin antibody on annexin A5 binding to endothelial cells. Manuscript, submitted

III. **Cederholm A**, Svenungsson E, Stengel D, Fei GZ, Pockley AG, Ninio E and Frostegård J. Platelet-activating factor-acetylhydrolase and other novel risk and protective factors for cardiovascular disease in systemic lupus erythematosus. Arthritis Rheum. 2004 Sep; 50(9): 2869-76

IV. Svenungsson E, **Cederholm A,** Jensen-Urstad K, Fei GZ, de Faire U and Frostegård J. Endothelial function and markers of endothelial activation in relation to cardiovascular disease in systemic lupus erythematosus. Manuscript, submitted

Abstract

Systemic Lupus Erythematosus (SLE) is a clinical syndrome of autoimmune origin. It manifests in diverse clinical and serological patterns and is considered to represent a prototype multisystemic autoimmune disease. The etiopathigenesis of SLE remains incompletely understood. Typical of SLE is a production of an array of autoantibodies, some of which are believed to be causative to the pathogenesis. Antiphospholipid antibodies (aPL), which are associated with arterial and venous thrombosis, are an example.

Cardiovascular disease (CVD), due to an underlying atherosclerosis and atherothrombosis, is the leading cause of death worldwide. Nowadays atherosclerosis is considered to be a chronic inflammatory disease, and evidence is accumulating for important role of immune mechanisms in atherothrombosis. During the last decade it has become evident that the incidence of premature cardiovascular disease is dramatically (up to 50 fold) increased in patients with SLE, emerging as the major cause of morbidity and mortality in this population. The link between manifestations of atherosclerosis/atherothrombosis such as coronary heart disease, ischemic cerebrovascular disease or peripheral arterial disease on the background of a systemic chronic inflammatory autoimmune disorder, such as SLE, is a subject of intensive research interest. We recently demonstrated that a combination of traditional and non-traditional risk factors including dyslipidemia, renal disease, lipid peroxidation, inflammation, and high levels of aPL characterize SLE-patients with CVD and that CVD in SLE is associated with atherosclerosis. This thesis work is aimed at studying potentially contributing autoimmune/immune mechanisms of atherothrombosis and atherosclerosis, as well as non-traditional risk and protective factors in SLE-related cardiovascular disease.

In the first paper, we study effects of plasma from twenty-six women with SLE and CVD (SLE cases), age-matched women with SLE and no history of CVD (SLE controls) and population controls (PC), on cultured human umbilical vein endothelial cells (HUVEC). We demonstrate that binding of Annexin A5, a plasma protein with putative antithrombotic effects, to HUVEC is significantly lower among SLE cases than SLE controls which in its turn is lower among PC. There were no differences in effects of plasma from the study groups on cell viability or apoptosis. Extraction of IgG from plasma restored Annexin A5 binding. Furthermore, absorption of aPL from plasma restored binding, suggesting that aPL play an important role. We hypothesize that decreased Annexin A5 binding to endothelium caused by aPL may be an important mechanisms causing CVD in SLE. We also demonstrate abundant presence of Annexin A5 within atherosclerotic lesions, especially at plaque sites prone to rupture.

In the second paper, we demonstrate that sera with high aPL titers and also a monoclonal antibody against an important phospholipid, a cardiolipin (aCL-mAb), both caused decreased Annexin A5 binding. Preincubation of intravenous immunoglobulin (IVIG) with high aPL titers plasma or with aCL-mAb restored Annexin A5 binding comparable to that of controls. This suggests that IVIG could play a therapeutic role in atherothrombosis, by neutralizing aPL. Still, when IVIG was added to normal healthy serum or to cell culture *per se*, a small decrease in Annexin A5 was observed. Recently, several reports have indicated that IVIG has CVD as a side effect in some patients, and our findings suggest a causative mechanism.

In the third paper, we compare emerging CVD risk factors including platelet activating factor (PAF) acetylhydrolase (PAF-AH) and soluble phospholipase A2 (sPLA2); heat shock protein (HSP)-related measurements and antibodies against endothelial cells (aEC-abs). Of all these measurements, only PAF-AH was associated with CVD in SLE. We hypothesize that PAF-AH promotes inflammation and atherosclerosis in SLE.

In the fourth paper endothelial factors in relation to CVD in SLE were studied. Endothelial function, as determined by flow-mediated dilatation (FMD) of the brachial artery, did not differ between SLE controls and population controls, suggesting that endothelial dysfunction and possibly CVD may not be a general feature of SLE but instead affect only a subgroup. This possibility should be further clarified in larger controlled prospective studies. SLE cases were not included since they were on nitro-related medications, precluding the FMD determination. Soluble levels of VCAM-1, and thrombomodulin (TM), both markers of endothelial cell activation/damage, where raised among SLE cases, suggesting that endothelial activation is one contributing factor to SLE-related CVD.

Taken together, in these studies a novel mechanism was demonstrated, namely an aPL-induced decreased binding of antithrombotic protein Annexin A5 to endothelium, that may play an important role in SLE-related CVD. We hypothesize that increasing Annexin A5 binding could represent a novel therapy against atherothrombosis, either by administration of Annexin A5, or by neutralizing aPL when present, with neutralizing antibodies from IVIG. Of note, IVIG may also *per se* have prothrombotic effects through this mechanism. PAF-acetylhydrolase activity and endothelial cell activation may also play a role in promoting CVD and atherosclerosis in SLE.

Keywords: systemic lupus erythematosus, cardiovascular disease, atherosclerosis, atherothrombosis, antiphospholipid antibodies, Annexin A5

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Abbreviations

ANXA5	annexin A5
APS	antiphospholipid syndrome
aPL	antiphospholipid antibodies
aCL	anti-cardiolipin antibodies
ACR	American College of Rheumatology
ANA	anti-nuclear antibodies
CVD	cardiovascular disease
CRP	C-reactive protein
ELISA	enzyme-linked immunosorbent assay
EBV	Epstein Barr virus
FMD	flow mediated dilatation
HUVECs	human umbilical venous endothelial cells
HSP	heat shock protein(s)
IMT	intima media thickness
IFN	interferon
IVIG	intravenous immunoglobulins
LAC	lupus anticoagulant
LDL	low-density lipoprotein
MI	myocardial infarction
PAF-AH	platelet activating factor- associated hydrolase
PS	phopshatidylserine
TF	tissue factor
TNF	tumor necrosis factor
TM	thrombomodulin
VCAM-1	vascular cellular adhesion molecule-1
SLE	systemic lupus erythematosus

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Introduction

Cardiovascular disease (CVD) due to an underlying atherosclerosis and atherothrombosis is the leading cause of death worldwide. During the last decade it has become evident that the incidence of premature cardiovascular disease is dramatically (up to 50 fold) increased in patients with systemic lupus erythematosus (SLE), emerging as the major cause of morbidity and mortality in this population. The fascinating link between exaggerated incidence and unusually early manifestations of atherosclerosis/ atherothrombosis such as coronary heart disease, ischemic cerebrovascular disease or peripheral arterial disease on the background of a systemic chronic inflammatory autoimmune disorder, such as SLE, is a subject of intensive research interest.

This thesis work is aimed at studying potentially contributing autoimmune/immune mechanisms of atherosclerosis and atherothrombosis, and non-traditional risk and protective factors in SLE-related cardiovascular disease.

Systemic Lupus Erythematosus

Definition

Systemic Lupus Erythematosus (SLE) is a clinical syndrome of an autoimmune origin, characterized by production of a wide array of autoantibodies, some of which are believed to be causative to the pathogenesis. SLE manifests in most diverse clinical and serological patterns and is considered to represent a prototype multisystemic autoimmune disease.

Historical and poetic aspects

In a long history of documented human suffering, perhaps the first original description featuring lupus is dated by the XIII century, when Rogerius had commented on cutaneous ulcerative eruptions resembling an omnious wolf's (Latin, *lupus*, wolf) bite. Although morphologically similar lesions could be inflicted by foreign assaulters, particulary by

Mycobacteriae, they could as well be a consequence of illicit tissue damage caused by one's own immune system. The poetic and rather compassionate allegory of lupus with the creatures of the animal kingdom continued when von Hebra in 1845 published illustrations depicting lupus-typical facial skin rash, ascribing a shape of it to a butterfly, spreading it's red (Gr. *erythema,* flash) wings over the mid-face. Later the term *erythematosus* in combination with lupus was applied by Cazenave (Cazenave 1852). Systemic nature of the disease was recognized around 1872, when M. Kaposi appreciated it's disseminated features (Kaposi 1872), the notion further supported on the turn of the XX century by W. Osler (Osler 1895). Contributive efforts of pathologists, especially in the 1920s and 1930s, attentively describing autopsy series with lupus- characteristic features, for ex. Libman-Sacks endocarditis (Libman 1924), lead to a firmly established clinical entity of systemic lupus erythematosus (Smith and Cyr 1988).

The description of the "LE cell" in the bone marrow of SLE patients (Hargraves 1949) originally rendered pathognomonic of lupus, only to be later identified in other autoimmune conditions, was followed by the fundamental recognition that some gammoglobulins were in fact targeting normal body components and tissues (Holman and Kunkel 1957). Importantly, introduction of the immunofluorescent techniques (Friou 1957) allowed detection anti-nuclear autoantibodies (ANA) in routine clinical work. With availability of mouse strains that develop lupus-like phenotypes (Hahn 2001) and other advances in cellular and molecular technology, intensive efforts in SLE research are continued with a potential promise that understanding the complexity behind SLE could lead to understanding of autoimmunity in general.

It is impressive how the minds of notoriously pragmatic scientists, fueled perhaps by frustration and even admiration for the so far unresolved fascinating puzzle behind this highly complex condition, are evoked to undertake poetic observational excursions. After the wolf and the butterfly, and even a Greek monster Hydra (Isenberg 1990) a yet another reminiscent metaphor was used by Alacron-Segovia (Alacron-Segovia 1999) when the complexity of SLE was compared to the Russian horse carriage-the Trojka, where the driving forces of three strong creatures (the "genetics", the immune system and the "unknown"), each one in its own right and, more importantly, in combination, are crucial to progress (of the disease) forward.

Epidemiology of systemic lupus erythematosus

SLE is a fairly common disease, with estimated prevalence to be about 60 per 100 000 inhabitants and incidence reported to be 4.8 per 100 000 in Sweden (Stahl-Hallengren, Jonsen et al. 2000), reflecting similar situation in European countries. It's racial, geographic, and even socioeconomic predilections come best to light in the USA, where prevalence rates have been reported to vary from 15 to 124 per 100 000, which apart from methodological differences, presumably reflect heterogeneity of genetic heritage or environmental exposures and/or access to diagnostic facilities (McCarty, Manzi et al. 1995).

SLE predominantly affects women of reproductive age, with the peak age of onset ranging between 20 and 40 years (Hahn 2001), pediatric and late onset SLE have been described as well (Klein-Gitelman 2004). The gender difference in prevalence of lupus during child-bearing age is in favor of females over males at a 1:9 ratio. It still prevails during pre- and post-menopausal years although not as dramatic, with the male-female ratio reduced to 1:3 (Hahn 2001).

Systemic Lupus Erythematosus – an autoimmune disease

SLE is a classic example of a multisystemic autoimmune disease and it is a clinical diagnosis unifying a number of heterogeneous syndromes. A multitude of interactions and yet undetermined triggers on a specific genetic background are responsible for shifting a physiological balance of previously "apparently healthy" immune system into a damaging aggressive mode, destructive to the host tissues and ultimately to the individual, resulting in manifested disease.

Autoimmunity

Autoimmunity, as defined by the presence of auto-reactive lymphocytes and antibodies, is a physiological inherent feature of the human immune system (Wardemann, Yurasov et al. 2003; Janeway 2005). As well as there is a number of commonly encountered circumstances (for ex. aging, malignancies, infections and immunodefiencies (Amital

1999)) where, although present, autoimmune phenomena are not necessarily associated with an overt autoimmune disease. Natural antibodies, germline encoded low-affinity polyspecific antibodies of predominantly IgM isotype, are capable of binding self-structures (Ochsenbein, Fehr et al. 1999). However, even IgG autoantibodies, products of affinity maturation and somatic selection events, are detectable in some asymptomatic individuals up to a decade before they develop a full-blown SLE (Arbuckle, McClain et al. 2003), again demonstrating that merely a presence of an autoantibody does not necessarily evoke a pertinent disease.

The state of immunologic tolerance, in which an individual is incapable of developing a damaging immune response to a specific antigen (host's own tissues, dietary proteins), is a physiologic prerequisite for homeostasis. Albeit not yet completely understood, the state of self-tolerance is believed to be developed and maintained by multiple controlling checkpoints, postulated to include clonal deletion, anergy and peripheral suppression (Cotran: 2005). Such central and peripheral controlling pathways must function perfectly in concert with each other in order to maintain a very delicate equilibrium between on the one hand an effective protection from foreign assaulters and on the other hand inflicting no damage in response to self-antigens. Consequently, a number of possibilities for emergence of pathological autoimmunity, with "breaking" of self-tolerance and manifested autoimmune disease, are given.

Inherent disturbances of immune system in particular those critical for T-cells homeostasis, such as mutations in genes coding for autoimmune regulator AIRE, participating in central thymic developmental processes, or a CTLA-4 receptor, providing an inhibitory co-stimulating signal or Foxp-3, a transcription factor required for development of regulatory T-cells, can lead to autoimmune human disease(Cotran: 2005). Additionally, dysfunctional idiotypic network has been suggested to contribute to autoimmune diseases by failing to control pathogenic idiotypes of autoantibodies (Amital 1999). Intrinsic phenomena, such as exposure of cryptic antigens or modifications of autoantigens could render them antigenic and trigger a destructive immune response (Janeway 2005).

The potential ability of infectious agents to unleash the immune system to destructive attack by shifting it away from itself, is known to include strategies of confusing the

immune system by virtue of cross-reactivity or molecular mimicry (McClain, Heinlen et al. 2005) or by inducing polyclonal activation of B cells (Granholm and Cavallo 1992) and possibly by leading to suppressor-T cells functional loss (Janeway 2005).

Autoimmune disease

Exclusively in the case when such self-reactive components of immune system inflict injury to self-tissues does it become an autoimmune disease. It is a fundamental recognition that the same factors that are effectors of innate and adaptive immune response to pathogenic microorganisms are causing autoimmune disease in predisposed individuals. Autoimmune diseases could be either organ- or structure specific, when immune system for example is attacking β-cells in pancreas as in insulin-dependent diabetes mellitus or acetylcholine receptors in skeletal muscles as in myasthenia gravis, or show more of a systemic disseminated character as in SLE, rheumatoid arthritis (RA) and systemic sclerosis. Interestingly, while habitually designated as the prototype multisystemic autoimmune disease, SLE would not fulfill the strict criteria for an autoimmune disease (Table 1).

Table 1. Criteria for an Autoimmune Disease
A defined circulating antibody or cell-mediated immunity to autoantigen
A defined specific autoantigen
The ability to produce the disease in an experimental animal model: 1. by passive transfer of the autoantibody / or autoreactive cells 2. by immunization with the autoantigen (with complete Freund's adjuvant)
The ability to generate the autoantibody or autoreactive cells after immunization with the autoantigen (with complete Freund's adjuvant)

Adapted from: Systemic Lupus Erythematosus, edited by R.G. Lahita, Academic Press (1999)

Ethiopathogenesis of systemic lupus erythematosus

Although currently written clinical textbooks are fair to state that "the cause of SLE remains unknown" and in clinical practice there is no cure for the disease (Hahn 2001) many emerging clues and concepts move us closer to de-puzzling abnormalities and chain reactions leading to diverse manifestation of lupus, to understanding what are the key players and what orchestrates the ethiopathogenesis of SLE (Figure 1).

Genetic components

Since the original observation of familial tendency in SLE (Leonhardt 1967), the search for genes involved in lupus ethiopathogenesis provided until now a rather expected but clear conformation that there is no single "SLE gene" solely responsible for either susceptibility, clinical presentation or activity of disease. Indeed, several chromosomal regions/genes were reported to confer genetic liability to SLE (Tsao 2003).

As products of different major histocompatibility (MHC) Class II alleles are displaying various abilities to bind and present peptides to lymphocytes (Marrack, Kappler et al. 2001), some alleles might be more predisposing to autoimmunity. Specific alleles of MHC Class II genes were reported to be associated with SLE in ethnic groups, for example increased frequency alleles encoding for HLA-DR3 was observed in Caucasians and in Japanese lupus patients stronger associations with HLA-DR2 were reported (Hahn 2001).

Lately, genetic studies to delineate the hereditary basis of SLE addressed the non-MHC genes, which could potentially alter intrinsic qualities of immune response and be involved in initiation and progression of disease. These include mainly genes, products of which are involved in 1). regulation of immune tolerance: such as co-stimulatory molecules-CTLA-4 (Fernandez-Blanco, Perez-Pampin et al. 2004) or programmed cell death 1 gene (PDCD1) (Prokunina, Castillejo-Lopez et al. 2002) 2). abberrant phagocytic activity together with deranged handling of immune complexes (IC) and residual apoptotic debris for example the early components of the complement system, FcγR family or mannose-binding lecitin (MBL)(Ip, Chan et al. 1998).

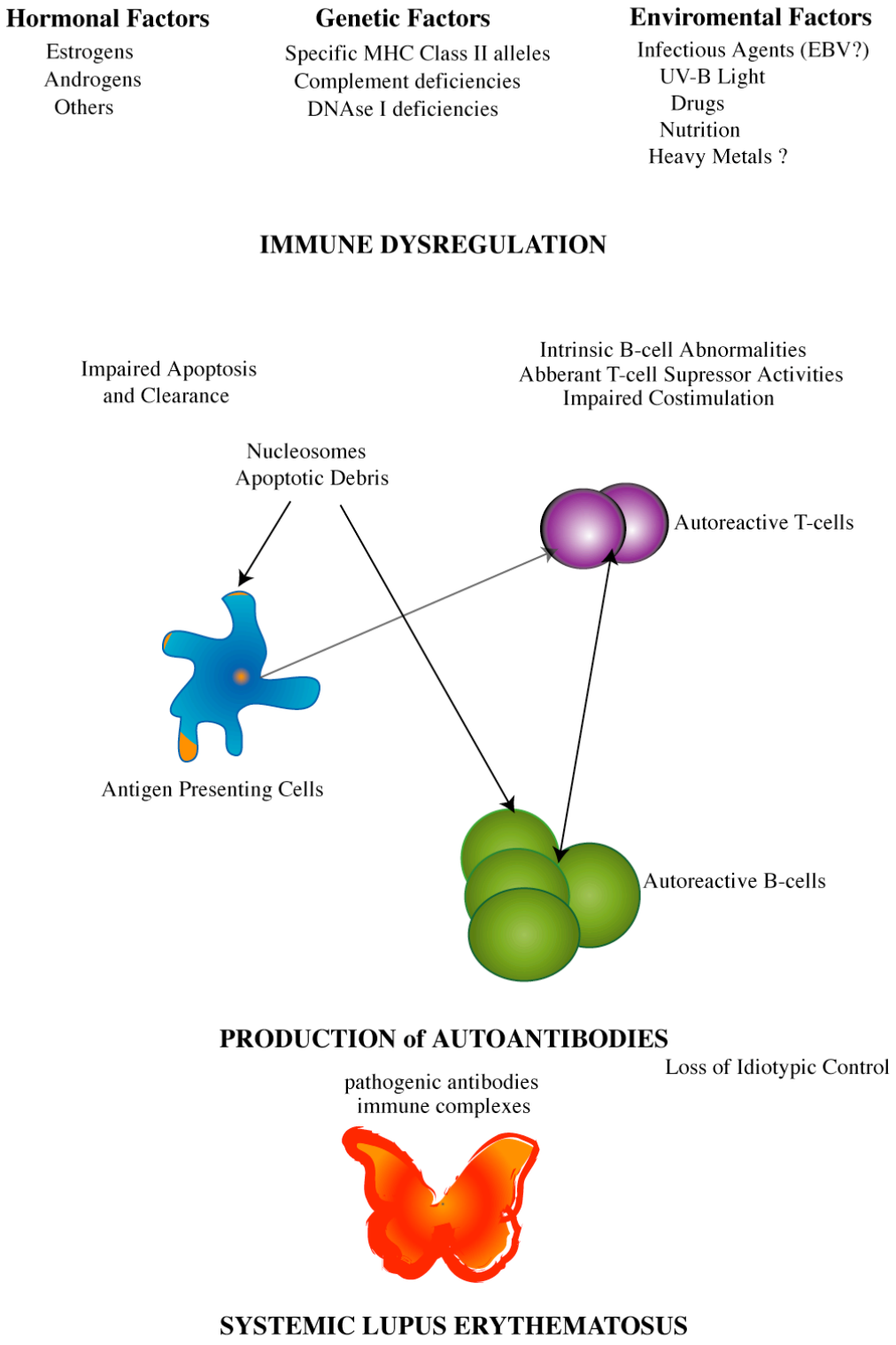

Figure 1. A possible model of ethiopathogenesis of systemic lupus erythematosus

Adapted from from Vasso S et al

Hormonal influences

As in other systemic autoimmune conditions, not exclusively in lupus, the relevance of hormones is suggested by the high prevalence of autoimmune diseases in females. Prevalence of SLE in women is highest during reproductive years, SLE has also been described in males with Kleinefelter's syndrome (Hahn 2001). Furthermore, administration of androgenic hormones as well as oophorectomy in a murine research models prone to spontaneous lupus-like disease, was shown to ameliorate the condition (Klein-Gitelman 2004). Interestingly and contrary to a common belief, in a recent well-conducted clinical trials administration of exogeneous estrogen and progesteron to post-menopausal SLE patients as hormone-replacement therapy (HRT), did not result in induction of significantly severe SLE flares (Buyon, Petri et al. 2005) neither had the use of oral contraceptives in pre-menopausal women (Petri, Kim et al. 2005).

Environmental factors

Cutaneous photosensitivity is a well-known phenomenon in SLE patients and exposure to sunlight has been described as a trigger of SLE flares (Meller, Winterberg et al. 2005).

Lupus-like disease was reported to be induced after nutritional exposures to alfalfa sprouts (Alcocer-Varela, Iglesias et al. 1985). Also certain pharmacological agents, for ex. D-penicillamine and hydralazine, are able to induce lupus-like transient illness. While the precise mechanisms are not yet defined, drugs (for ex. procainamide) are able to modify DNA by de-methylation (Cornacchia, Golbus et al. 1988), altering thereby it's structural properties and rendering it immunogenic. Exposure to heavy metals, such as mercury, could induce lupus-like syndromes in murine studies, however the relevance of occupational exposure to metals, but also to silica or organic solvents as trichloroethylene (TCE) to human lupus remains undetermined (Parks and Cooper 2005).

Apart from being recognized as the initiators of SLE flares, evidence is accumulating supporting the role of infections as initial triggers of SLE. Among the putative candidates, the B-cell trophic Epstein Barr virus (EBV) has recently re-emerged within the focus of interest as a suspect. Clinical case reports exist where the onset of SLE immediately follows an acute EBV infection (Dror, Blachar et al. 1998), epidemiological

studies demonstrate elevated EBV load (James, Neas et al. 2001) and antibodies to EBV proteins (Moon, Park et al. 2004) in SLE patients. Linking EBV and SLE on molecular level, there is evidence for molecular mimicry between EBV proteins (such as Epstein-Barr virus nuclear antigen (EBNA-1)) and Ro (McClain, Heinlen et al. 2005) or Sm (Sabbatini, Bombardieri et al. 1993), both autoantigens implicated in SLE.

While EBV is able to directly infect and activate B-cells, alternative mechanisms might be able to promote activation of B-cells, namely via interactions of microbial products with the components of innate immune system. Recent findings suggest that a pattern recognition receptor of innate immune system, a toll-like receptor 9 (TLR9*),* recognizing CpG motifs of hypo-methylated DNA, characteristic of bacterial and viral DNA, can signal B-cell activation and initiate class switch DNA recombination and may therefore facilitate the production of pathogenic autoantibodies (He, Qiao et al. 2004).

Immune system disturbances

What have we learned from the "mighty mouse"(Hahn 2001)?

Several murine models, such as the New Zealand Bielschowsky black mice (NZBW), or MRL-Fas (lpr) mice strains that spontaneously develop lupus-like disease, have added to our understanding of autoimmune dysregulation in lupus. Their anti-DNA responses are similar in frequency and specificity to that found in humans (Theofilopoulos and Dixon 1985). The murine major histocompatibility complex genes act as major genetic elements contributing to the murine lupus susceptibility (Ibnou-Zekri, Iwamoto et al. 2000). In the course of murine lupus, anti-nucleosomal antibodies appear early, before the anti-ds-DNA and anti-histones antibodies emerge, supporting therefore speculation that nucleosome is the initial driving autoantigen in SLE (Koutouzov, Jeronimo et al. 2004). Abberations in the murine B-cell functions provide clues to the potentially important mechanisms, for instance B-lymphocyte stimulator (BlyS), which is able to sustain a survival signal for B-cells, can induce lupus-like phenotype in mice overexpressing BlyS (Mackay, Woodcock et al. 1999).

Cellular immune dysfunction in systemic lupus erythematosus

Multiple immunologic aberrations found in SLE patients (Cotran: 2005) leave little doubt there is a fundamental derangement of immune homeostasis. In the overall concept of SLE pathogenesis (Fox 2005), possibly the predisposition to impeded clearance of cellular debris and failure to deplete potentially autoimmune B-cells at the peripheral levels (Yurasov, Wardemann et al. 2005) are of importance.

The central effector role of autoreactive B-cells in the pathogenesis of SLE is emphasized by multiple species of secreted autoantibodies. The number of peripheral naïve B-cells is frequently reduced in SLE, while the plasma cells increase accordingly with disease activity (Odendahl, Jacobi et al. 2000). Both, polyclonal stimulation of B-cells (Granholm and Cavallo 1989; Granholm and Cavallo 1992) and clonal B-cells expansion driven by self-antigens might contribute to production of autoantibodes in SLE. However, molecular analysis of pathogenic autoantibodies for instance of aPLs (Lieby, Soley et al. 2003) strongly suggest the latter possibility. Furthermore additional possibilities exist how B-cells can contribute to SLE pathogenesis. A rather recently recognized but not less significant antigen-presenting function of B-cells (Janeway 2005) is able to sustain T-cell activation and maintain the vicious circle of autoimmunity

Correlation of lymphopenia with disease activity and presence of antibodies targeting various cellular antigens make it difficult to conclude whether the decline in T-cells, observed in SLE, is a contributive factor in immunoregulatory disbalance unique of SLE or whether it is due to the specific effect of anti-lymphocyte antibodies, since the majority of SLE patients develops such autoantibodies in the course of disease (Winfield, Fernsten et al. 1996).

According to a recent hypothesis, a Type I interferon, interferon–alpha (IFN-a), an important cytokine involved in many immunological reactions including anti-viral defenses may play an important role in SLE. Early findings have demonstrated that raised activity in the IFN-a system is common in SLE, and also that, when given therapeutically against infectious and malignant disease, IFN-a may evoke symptoms reminiscent with that of SLE. The majority of SLE patients show a pattern of IFN-a–inducible gene expression, the so-called "IFN signature". It is possible that IFN-a plays a role in SLE

and in autoimmunity in general by promoting loss of tolerance (Ronnblom, Eloranta et al. 2006).

Autoantibodies in systemic lupus erythematosus

SLE is characterized by production of an array of autoantibodies. In fact there is no other condition described, where more than more than 100 (!) various autoantibodies have been detected (Sherer, Gorstein et al. 2004). Their target specificities are non organ-specific and include nuclear, cytoplasmic, cell surface membrane antigens as well as plasma and matrix proteins. Traditionally, indirect immunofluorescent techniques are used to detect basic fluorescence patterns, suggestive of generic antinuclear antibodies (ANA), which are the hallmark of SLE. Presence of antibodies to double-stranded DNA (anti-ds DNA) and the so-called Smith (Sm) antigen is highly specific and virtually diagnostic for SLE. Some subsets of autoantibodies are reported to be associated with SLE flares or specific organ involvement.

Autoantibodies can induce damage either by non-specific immune complex deposition on bearers of Fc-receptors or by direct binding to the epitopes on the specific tissues and leading to antibody-dependent cell cytotoxicity (ADCC)-mediated cell lysis. While most of visceral lesions (for ex. glomerular renal damage) in SLE are mediated by immune complexes, it is also increasingly appreciated that autoantibodies can directly affect cellular functions or interfere with physiological pathways, such as coagulation cascade, by acting against coagulant factors. Neuronal dysfunction in SLE is perhaps an autoantibody-mediated syndrome, since the latest robust evidence causatively linking autoantibodies to CNS dysfunction, although in murine model, has been provided by Huerte et al. In their study autoantibodies were able to mediate neuronal death and affect emotional behavior after reaching brain tissues (through lipopolysaccharide-compromized blood-brain barrier) (Huerta, Kowal et al. 2006). It is tempting to speculate that these incredibly interesting results can be translated into the human lupus since emotional alterations, frank depression and psychosis are observed features of neuropsychiatric SLE (Table 3).

Refusing to die and perish with quiet dignity?

Apoptosis is a pathway of "dignified" cellular death, essential in physiologic turn-over and housekeeping. The precise apoptotic sequence executed by a dying cell is designed in order to disappear in a governed fashion without inflammation (Voll, Herrmann et al. 1997), quietly and beautifully as a falling leaf (Gr, *apoptosis*) (Kerr, Wyllie et al. 1972) as opposed to an explosive inflammatory nature accompanying necrotic death(Searle, Kerr et al. 1982). Undoubtedly, rapid and efficient phagocytosis and clearance of resulting apoptotic bodies and debri is crucial in order to fulfill the purpose.

The hypothesis, unifying aberrant apoptotic processes in SLE and autoantibody production deserves extra attention, as many autoantigens implicated in SLE, phospholipids and nucleosomes in particular, are sequentially exposed on the apoptotic cells and blebs (Casciola-Rosen, Anhalt et al. 1994). At the same time as cryptic antigens are exposed, variable modifications of proteins in association with apoptosis occur (Rosen, Casciola-Rosen et al. 1995) that may result in exposure of T and B cells to earlier "unknown" epitopes. Also one of the key targets of SLE-associated autoantibodies, nucleosomes, have been shown to be released during the early phases of apoptosis (van Nieuwenhuijze, van Lopik et al. 2003).

In a setting of SLE, impaired clearance of apoptotic material has been brought to attention by *in vitro* studies demonstrating diminished capacity of monocytes from SLE patients to phagocytosis (Herrmann, Voll et al. 1998). As well as by clinical observations, where deficiency in early complement component C1q predisposes to early-onset photosensitive SLE (Sontheimer, Racila et al. 2005), given that rapid clearance of released intracellular material in to the circulation in particular is assisted by opsonization by C1q in addition to CRP and/ or antibodies (Walport, Davies et al. 1998).

Recently, a DNAse-1 deficient mice is shown to develop a lupus-like phenotype (Napirei, Karsunky et al. 2000), a finding supporting a possible role of DNAse-1in humans is a reported lower activity of DNAse-1 in SLE patients (Chitrabamrung, Rubin et al. 1981). Implications of such findings possibly offer a plausible explanation that a specific hallmark of SLE could be a combination of increased and impaired apoptotic process and/or clearance of apoptotic material (Gaipl, Voll et al. 2005), driving the autoantibody production by the excess burden of otherwise cryptic auto-antigens.

Cytokines in systemic lupus erythematosus

Cytokines are soluble protein mediators, acting as highly effective means of communication between cellular components of the immune system. A popular immunological concept, albeit most likely an oversimplified one, make use of subdividing the cytokines into two major subsets based on the main sources of production accordingly. That is into those produced by distinct CD4+ T-lymphocytes subsets, namely Th1 or Th2 or more of functional subdivision into anti- or pro- inflammatory cytokines. Based on available evidence, as to the levels of circulating cytokines or various *in vitro* studies, several authors argument in favor of dichotomizing autoimmune conditions according to their dominant dependence on Th1 or Th2 cells (Amital 1999), while others openly challenge such concept (Theofilopoulos, Koundouris et al. 2001). One of the major and quite justifiable criticisms is that most, if not all, patients are under one or another way of immunomodulating and immunosuppressive treatments, making the available evidence hardly conclusive. Yet there is little controversy in that both Th-1 and Th-2 cytokines are participating in SLE evolution (Hahn 2001).

Clinical Features of Systemic Lupus Erythematosus

Given its protean systemic nature, SLE can affect any and every organ or system of the body, hence the boarders are stretched out to virtually all clinical specialties. A patient with SLE can present with a subtle indolent skin rash to a dermatologist, be assessed for unexplained fever or fatigue by a general practitioner or found in dramatic circumstances under intensive care with fulminant life-threatening multiple organ failure. Although some physicians would entertain an idea of rendering certain SLE cases a “diagnosis, which can be made on the doorsteps”, given the presence of characteristic malar rash facial lesions, SLE remains a challenging condition to diagnose.

The underlying multilayered complexity of SLE becomes evident by glancing over the criteria for definition of SLE cases provided by the American College of Rheumatology (ACR) (Table 2). Noteworthy is that the primary purpose of ACR guidelines was to

establish a certain degree of standardization for research purposes and they were not intended to become a diagnostic tool.

Table 2. The 1982 American College of Rheumatology Criteria for Classification of SLE	
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences
2. Discoid rash	Erythematosus raised patches with adherent keratotic scaling and follicular plugging
3. Photosensitivity	
4. Oral ulcers	Includes oral and nasopharyngeal observed by physician
5. Arthritis	Non-erosive arthritis involving two or more peripheral joints, characterized by tenderness, swelling, or effusion
6. Serositis	Pleuritis or pericarditis, rub or evidence of pericardial effusion
7. Renal disorder	Proteinuria >0.5 g/d or >3+, or cellular casts
8. Neurologic disorder	Seizures without other cause or psychosis without other cause
9. Hematologic disorder	Hemolytic anemia or leucopenia (<4000/ml) or lymphopenia (< 1500/ml)
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 ANAs by immunofluorescence or an equivalent assay at any point in time in the absence of drugs known to induce ANAs

If four of this criteriae are present at any time during the course of disease, a diagnosis of systemic lupus erythematosus can be made with 98 percent specificity and 97 percent sensitivity.
Source: (Tan, Cohen et al. 1982); * detectable aPL or LAC was added in 1997 (Hochberg 1997)
Sm: the Smith antigen, LE: lupus erythematosus cell, VLDR: Venereal Disease Research Laboratory, ANA: anti-nuclear antibodies, aPLs: antiphospholipid antibodies, LAC: lupus anticoagulant activity.

The course of the disease is variable and mostly unpredictable. Several instruments have been developed for the assessment of disease activity and/or clinical response, such as SLEDAI, BILAG and SLICC (Liang, Socher et al. 1989). Nevertheless there are few biomarkers of value to predict susceptibility, to allow undoubtful diagnosis or to monitor disease activity and response to treatment that can be used in clinical practice (Liu, Manzi et al. 2005). Clinical features associated with SLE are listed in Table 3.

Table 3. Clinical manifestations of Systemic Lupus Erythematosus

Systemic	Fatique, fever, weight loss
Musculoskeletal	Arthralgias, myalgias, non-erosive polyarthritis
Cutaneous	Malar rash, photosensitivity, ulcerations
Neurologic	Cognitive disturbances, psychoses, seizures, headaches
Hematologic	Leukopenia, thrombocytopenia, lymphopenia, hemolytic anemia
Cardiopulmonary	Pleurisy, pericarditis, endocarditis (Libman-Sacks), premature cardiovascular disease
Renal	Proteinuria, nephrotic syndrome
Thrombosis	Arterial and venous
Gastrointestinal	Non-specific anorexia, nausea, diarrhea
Other	Recurrent fetal loss, sicca syndrome

Current perspectives and the future of treatment

Until the recognition of value of corticosteroids and their introduction in clinical practice in 1950s, SLE was considered to be a dreadful fatal disease with a non-predictable course. Intensive research in the area of SLE pathogenesis, until now, all progress aside, has not given us a clear understanding as to the main mechanisms triggering and driving the disease. Not surprisingly there is still no "cure" for SLE.

Nowadays corticosteroids in combination with other immunosupressive and immunomodulating agents (hydroxychloroquine) are currently used as a maintenance therapy, with more aggressive regimens (cyclophosphamide and azathioprine) added to tame the immune system during exacerbations of the disease. Such treatment regimens have significantly improved the SLE outcome, with expected survival approximately 90% in 5-years and 80% in 10-years (Klein-Gitelman 2004). As a corticosteroid-sparing strategy, a weak androgenic steroid (dehydroepiandrosterone) has been used, although in that particular clinical trial it failed to show significant effect (Petri, Lahita et al. 2002). In cases of severe treatment-refractory SLE, radical attempts to reverse the immune dysregulation associated with SLE, using immune ablation and a follow-up rescue via autologous hematopoetic stem cells transplantation has not been all too promising (Traynor, Barr et al. 2002; Lisukov, Sizikova et al. 2004).

Nevertheless, the focus of treatment of SLE is shifting from the broad non-selective immunosupression to more targeted therapies, hopefully able to control SLE-relevant immune processes. The undeniable success of a new generation of drugs, the so-called "biologics" (Andreakos, Foxwell et al. 2002), resembling endogeneous molecules, leaves us with hopeful notes. For many patients suffering from rheumatoid arthritis (RA), who are cared for in a health system, which is able and willing to afford high concomitant expenses, the promise of, if not cure, but of rapid improvement, seem to be fulfilled with either a monoclonal anti-TNF antibody or a soluble TNF-receptor, antagonizing circulating TNF, crucial in RA pathogenesis, notably revolutionizing once more since the introduction of corticosteroids, the treatment of RA. Anti-TNF treatment has not been properly evaluated in the setting of SLE, although a very small series of SLE patients with nephritis were reported to improve with anti-TNF treatment (Aringer, Graninger et al. 2004).

In SLE targeting of B-cells populations by depleting anti-CD20 chimeric monoclonal antibody (Rituximab) has been promising in reducing disease activity (Anolik, Barnard et al. 2004; Looney, Anolik et al. 2005).

Antiphospholipid antibodies

The first serological abnormality associated with SLE had been in fact a phenomenon of false positive test for syphilis caused by antiphospholipid antibodies (aPL) (Wasserman 1906). Another known aPL-related phenomenon, namely lupus anticoagulant (LAC) (Feinstein and Rapaport 1972), was thought to be a mere laboratory curiosity for a more than 50 years. The mystery behind the LAC phenomenon, causing prolongation of clotting times in vitro, has been partly unveiled by Thiagarajan et al in 1980 (Thiagarajan, Shapiro et al. 1980) when direct indications were presented that antibodies responsible for LAC activity *in vitro* were in fact recognizing phospholipids (PL). These findings shed light on why patients with positive LAC but no syphilis were chronically positive in the Venereal Disease Research Laboratory (VLDR) test for syphilis, where the main antigen, resembling the surface structure of *Treponema pallidum*, was extracted from bovine hearts and was shown to be indeed an anionic phospholipid, a cardiolipin (CL) (Pangborn 1941).

The broad range of aPLs specificities is now known to include anionic phospholipids (such as cardiolipin, phosphatidylserine, phosphatidylglycerol, phosphatidylinositol), their putative protein cofactors such as β_2-GPI (beta2-glycoprotein I) (Galli, Comfurius et al. 1990; McNeil, Simpson et al. 1990), prothrombin, or a complex between PL and a protein (Bertolaccini, Hughes et al. 2004). Cardiolipin, being a component of inner mitochondrial membrane, was considered a cryptic antigen, therefore not readily accessible to aPLs. Recently, Deguchi et al (Deguchi, Fernandez et al. 2000) challenged this notion by specifically searching for and identifying CL in circulation of healthy adults and concluded that CL was a major anionic phospholipid in human plasma, selectively enriched in low density lipoprotein (LDL).

aPLs can be natural or induced antibodies, they are detected in general population (Shi, Krilis et al. 1990) or patients with infections, mostly viral (Colaco, Mackie et al. 1989) as HIV, EBV but also in parasitic malaria and chronic tuberculosis and lepra (Facer and Agiostratidou 1994). Hematologic disseminated malignancies are associated with aPLs, also in aging population the titers are enhanced (Asherson 1999).

The antiphospholipid syndrome

aPLs are thought to be causative to the pathogenesis of the antiphospholipid (APS) syndrome (Levine, Branch et al. 2002), defined as an association of clinically manifested thrombosis, either arterial and venous, recurrent pregnancy loss and thrombocytopenia with persistent (more than 2 positive findings at least 6 weeks apart) elevation of routinely determined aPLs (aCL and/or LAC) (Wilson, Gharavi et al. 1999).

The antiphospholipid syndrome is a fairly young clinical entity, gradually recognized and formally coined into the term "APS" by G.Hughes (Harris 1987). APS might be "primary" (Asherson, Khamashta et al. 1989), as the only feature, without an underlying disease or "secondary", if associated with SLE or with other non-lupus diseases (Asherson 1999). Among SLE patients, approximately 30-50% have detectable aPLs and up to half of them develop APS (Petri 2000). If presented in it's more fulimnant course, when arterial and/or venous thrombosis occurs within days at multiple sites, it is named a "catastrophic APS" (Asherson and Cervera 2000).

The pathogenicity of antiphospholipid antibodies

Considerable interest in aPLs is explained by strong epidemiologic data linking aPLs with thrombotic events and understanding beyond doubts that some aPLs are indeed pathogenic (Levine, Branch et al. 2002). However, the methodology of aPLs determinations (both in clinical and research settings), despite international efforts and recommendations (Harris, Gharavi et al. 1987; Harris, Pierangeli et al. 1998), is only slowly becoming accepted and implemented. Hence it has been difficult to draw conclusions based on clinical studies, as some report associations between aPL and CVD in SLE (Svenungsson, Jensen-Urstad et al. 2001; Ames, Margarita et al. 2002) while others do not (Petri 2004). Similary, conflicting evidence regarding an association link between CVD and aPLs (aCL) in general population without over autoimmune disease can be demonstrated by Lancet publications in 1990's, when presence of aCL antibodies have been reported by Hamsten et al (Hamsten, Norberg et al. 1986) to be a risk for subsequent CVD events in young MI survivors, only to be challenged later by Sletnet et al (Sletnes, Smith et al. 1992).

An overview of aPL-related literature leaves one in a state of confusion and I can not resist but cite Lieby et al (Lieby, Soley et al. 2003) for a reflective summary "despite intensive efforts trying to understand the aPL-thrombosis association, it is somewhat surprising that we remain so ignorant about the origin of these antibodies in healthy individuals and in autoimmune patients, as well as about the mechanism of their pathogenicity in patients".

Nevertheless, there is substantial experimental evidence pointing at rather multiple and non-mutually exclusive possibilities behind procoagulant effect of aPLs. Besides their interference with soluble coagulation factors (Esmon, Safa et al. 2000), they are able to activate endothelium (Simantov, LaSala et al. 1995), induce expression of tissue factor (TF) on endothelial cells (Vega-Ostertag, Casper et al. 2005) or monocytes (Reverter, Tassies et al. 1998) and were shown to interfere with annexin A5 protective "shield" on throphoblasts in placental microcirculation (Rand and Wu 1999). Therefore focus of research in the field of APS has been shifting from the aPLs-induced "coagulopathy" to "endotheliopathy" (Amengual, Atsumi et al. 2003).

Still many crucial clinical questions remain unanswered. It is not clear whether the thrombotic risk is determined and or/predictable by a specific type, titer or isotype of aPLs. These are not trivial issues for clinical practice as preventive treatment is not easy to administer, given that no predictions can be made and fear of recurrent thrombotic events lead to decisions to keep patients on anticoagulant treatment indefinitely.

Annexin A5

Annexin A5 (ANXA5) belongs to a family of highly conserved ubiquitious proteins, the annexines (Gerke, Creutz et al. 2005) (Gr, *annex-* to hold together), with 12 members (ANXA1 to ANXA13) currently recognized in vertebrates (Gerke and Moss 2002). Although a unique set of physiological functions is yet to be assigned to each individual protein, generally, the hallmark of their functional activity involves interactions with cellular membranes. Annexines have been implicated in signal transductions, organization of cytoskeleton and endocytosis (Gerke, Creutz et al. 2005).

ANXA5, previously known as vascular anticoagulant alpha (VAC-α), placental anticoagulant protein I (PAP-I) or annexin V, had been originally purified from anticoagulant fraction derived from human umbilical cords (Reutelingsperger, Hornstra et al. 1985) and placenta (Funakoshi, Heimark et al. 1987), rich sources of ANXA5. Otherwise ANXA5 is widely distributed in human tissues, predominantly in intracellular compartments. Soluble form of ANXA5 is detectable in plasma of healthy population at a range of 0.6-28 ng/ml (Gonzalez-Conejero, Corral et al. 2002), as well as in cerebrospinal fluid(Vermes, Steur et al. 1999) and urine (Matsuda, Kaneko et al. 2000). A close structural homology of membrane-bound and secreted ANXA5 forms suggest their similar functional activity (Huber, Romisch et al. 1990).

Functions of ANXA5

Since the ability of ANXA5 to act as an instantaneous anticoagulant was observed by Reutelingsperger et al (Reutelingsperger, Hornstra et al. 1985) *in vitro*, multiple investigators have contributed to the understanding of the mechanisms behind ANXA5 effect. One of the main biochemical characteristics is its high affinity to anionic phospholipids in a Ca^{2+}-dependent manner. Therefore, it is generally believed that the main anticoagulant effect exerted by extra-cellular ANXA5 *in vivo* can be attributed to its high affinity for anionic phospholipids (mainly phosphatidylserine; PS). By forming a symmetrical crystal cover in a protective "shield"-like fashion (Rand and Wu 1999) over membrane patches expressing PS, ANXA5 might indirectly interfere with assembly of prothrombinase (as demonstrated by 99% inhibition of prothrombinase activity *in vitro*) and tenase complexes, simply by mechanistic increase in membrane rigidity (Andree, Stuart et al. 1992). However, more specifically, ANXA5 crystallization might lead to opening of a new cell entry "portal" (Kenis, van Genderen et al. 2004) and lead to downregulation of surface expressed tissue factor (TF) via endocytic internalization (Ravassa, Bennaghmouch et al. 2005).

The concept of a coagulation cascade usually accentuates chain reactions involving interactions between soluble pro- and anti-coagulant factors. Whereas during *in vivo* initiation and amplification of thrombus formation, the activity of soluble factors is crucially dependable on availability of cofactors (anionic phospholipids, such as PS and

Ca^{2+}), which are provided by cellular surfaces of endothelial cells, platelets and microparticles (Hoffman 2003). Under physiological conditions, the density of surface anionic phospholipids (mainly PS) is not sufficient to support initiation of the clotting cascade (Esmon 2000), which changes rapidly in association with activation/and or injury. For instance, endothelial cells expose surface PS in response to viral infections (van Geelen, Slobbe-van Drunen et al. 1995), hyperlipidemia (Lupu, Moldovan et al. 1993) and not, as previously believed, as an exclusive feature of early apoptosis. Procoagulant membrane components of platelets (PLT) include PS and sulfatide, a glycosphingolipid, believed to be responsible for Factor XII activation (Kyogashima 2004). Sulfatide is a yet another recently recognized ligand for ANXA5 (Ida, Satoh et al. 2004) and, not surprisingly, a target for aPLs (Merten, Motamedy et al. 2003). A newly appreciated source of procoagulant surfaces are microparticles, microvesicles shed into circulation by activated endothelium, PLT and leukocytes alike. Microparticles from multicellular sources carry phospholipids and TF on membranes, contributing to the net increase of procoagulant surfaces and are associated with an increased risk of thrombotic events (Eilertsen and Osterud 2005).

ANXA5 and antiphospholipid antibodies

In fact, a recent introduction of the term "annexinopathies"(Rand 1999), is partly based on a growing recognition that ANXA5 has a significant role in the physiology of placenta and is important for successful pregnancy. Rand et al (Rand 2000) have proposed that in a setting of antiphospholipid syndrome, an aPL-mediated disruption of the protective anticoagulant ANXA5-shield leads to microthromboses and consequently placental insufficiency, resulting in miscarriage. However, in addition to aPLs-induced placental microthromboses, both activated complement (Girardi, Redecha et al. 2004) and TNF-α (Berman, Girardi et al. 2005) have been discussed as factitious effectors of fetal damage, ultimately leading to pregnancy loss.

ANXA5 in cardiovascular disease

ANXA5 plasma levels were found to be markedly elevated in response to cardiac ischemia in animal models (Kaneko, Matsuda et al. 1994) and significantly elevated in

humans in early MI before any detectable increase in creatine kinase (CK) levels (Kaneko, Matsuda et al. 1996). Given that ANXA5 is not exclusively present in cardiomyocytes and was shown to be released from a multitude of cells during traumatic injuries, its low specificity has precluded its potential to become a new clinically useful marker in acute coronary syndromes (Peetz, Hafner et al. 2002). Nevertheless, recently interest in a subject of soluble ANXA5 has intensified once again, triggered by a report by Gonzales et al. (Gonzalez-Conejero, Corral et al. 2002). They presented evidence that a common polymorphism, AnnxA5-1C/T, in the so-called Kozak sequence in the ANXA5 gene, was associated with significantly decreased risk for MI in young men of Mediterranean origin, putatively via enhanced translational efficiency and therefore increased (protective) ANXA5 levels in circulation. Since than at least two studies have not been able to confirm such association (Kenis, Doggen et al. 2003; Kaikkonen, Kakko et al. 2005).

Interestingly, increased levels of ANXA5 were significantly associated with increased post-MI mortality within the first 24 hrs (Gonzalez-Conejero, Corral et al. 2002). One could speculate that higher ANXA5 levels post-MI might indeed reflect the extent of myocardial ischemic damage and leaking necrotic cardiomyocytes being the source of it. More intriguing is the possibility that ANXA5 had been prevented from properly exerting its antithrombotic function in these patients without overt autoimmune disorders by for ex. transiently enhanced aPL. Clinical observations and epidemiological data confirm increased risk of MI in association with infections (Meier, Jick et al. 1998) and several common infections are associated with induction of aPL, although their pathogenic prothrombotic potential is not clearly defined (Asherson 1999). However, our preliminary data shows that for ex. antibodies of IgG subclass against *S.pneumoniae* have a potential to decrease ANXA5 binding to endothelium *in vitro* (Cederholm A, 2005, unpublished)

ANXA5 a new " wonder weapon in antithrombotic arsenal"(Cederholm and Frostegard 2005)?

In animal models, a bolus infusion of ANXA5 has been demonstrated to be highly efficient in prevention/inhibition of thrombus formation *in vivo* even after an extensive mechanically inflicted injury of a vessel wall (Thiagarajan and Benedict 1997), another

study (Ju, Wang et al. 2004) has compared ANXA5-derivate with heparin and found ANXA5 being a significantly superior antithrombotic agent as measured by wet and dry weights and lenghts of stripped thrombi, whereas its anticoagulant activity was comparable to that of heparin. A fusion constructs between ANXA5 and the so-called Kunitz-type protease inhibitor (TF pathway inhibitor) targeting simulataneously PS and TF have been tested in a murine model by Chen et al (Chen, Vicente et al. 2005), demonstrating up to 4 fold increase in antithrombotic activity of the construct. The effect of ANXA5 as a better antithrombotic agent perhaps could be explained by a greater relevance of platelets in formation of arterial thrombus. Processes of activation, adhesion of platelets as well as circulating procoagulant microparticles, are associated with exposure of known ANXA5 ligands: PS and sulfatide. *In vitro* ANXA5 has been shown to efficiently inhibit aggregation of platelets and their fusion with TF-bearing microparticles (Del Conde, Shrimpton et al. 2005). Also in a venous injury animal model, ANXA5 was found to accumulate selectively at the site of injury, inhibiting thrombin generation locally without displaying a generalized state of hypocoagulation (Van Ryn-McKenna, Merk et al. 1993)

However, a lack of any specific phenotype in a recently generated ANXA5 knock out mice (Brachvogel, Dikschas et al. 2003) was surprising. The interpretation of this model is difficult as the issues of compensatory up-regulation and pleiotropic effects of other annexines, such as ANXA4 and/or ANXA6, sharing close structurally homology with ANXA5, currently remain unresolved.

Annexin A5 has become a subject of my personal interest and scientific affection throughout this PhD-related work (reviewed by (Cederholm and Frostegard 2005). I am convinced the antithrombotic potential of ANXA5 deserves further attention and studies in order to determine its true physiologic impact and possible therapeutic implications.

Cardiovascular Disease

The annual World Health Organization reports on morbidity and mortality demonstrate year after year that the leading cause of death in the industrialized nations is hiding behind an umbrella terminology "cardiovascular disease". Myocardial infarction (MI) accounts for highest morbidity and cerebrovascular events (including an ischemic stroke) for highest morbidity rates (Rodgers 2002). Although still remarkably devastating in former Soviet Republics, Scotland and Finland, the incidence rates of acute CVD have been reported to be decreasing in the Western world. Partly due to aggressive educational efforts in terms of both risk factor modification and timely symptom recognition by layman, overall improved treatment guidelines and available effective therapeutic options. However, prognosis for the next twenty-year period is evidently less optimistic for the parts of the world not commonly associated with high rates of CVD, such as Asia, South America and Japan (Gaziano 2005).

Cardiovascular disease and atherosclerosis

Manifestations of CVD after the 4th decade of life are caused predominantly by the underlying acquired vascular pathology, an atherosclerosis and the atherothrombotic consequences thereof. This is the most prevalent clinically significant arterial pathology, affecting in a lifetime, to a various degree, nearly every individual. Atherosclerosis belongs to a group of disorders, arterioscleroses, characterized by the hardening of the arterial wall (Gr, *sclerosis*, hardening) and loss of its elasticity. Terminology *atherosclerosis*, in addition to the hardening of the wall, implies presence of a greasy substance within the vascular wall (Gr, *atheroma*, greasy tumor). Atherosclerosis targets mainly elastic and muscular arteries, with aorta, coronary and cerebral arteries being primarily subjected to this disease. Clinical consequences can be caused by atherosclerosis by either an acute thrombotic/or embolic obstruction or, less commonly, by a slow progressive narrowing of the vessel and subsequent critical ischemia of the tissues within the perfusion area of the affected artery. The death toll is taken by atherosclerosis with MI, ischemic stroke, sudden cardiac death, chronic ischemic heart

disease, and ischemic encephalopathy, mesenteric, renal or distal limbs ischemia. Additionally, structural alterations associated with atherosclerosis are detrimental in large elastic vessels such as abdominal aorta by weakening vessel walls, predisposing to dilatation, aneurysms, and catastrophic ruptures.

The atherosclerotic plaque

The basic atherosclerotic lesion is the plaque (Figure 2), which comprises a focal elevation within intima, consisting of a fibro-fatty core accumulation covered by a fibrous cap. In a very simplistic view, the major components within the "build up" of a plaque are macrophages, foam cells, T-cells, smooth muscle cells as well as cholesterol esters, calcifications and accumulated debris embedded in matrix components.

There are different plaque types in regard to their composition complexity, size and developmental stages. The American Heart Association had formed a Working Task Force to make the definition more refined and clear by introducing the nomenclature of four (I-IV) stages of plaque, based on the gross morphology and histopathological evaluation (Stary, Blankenhorn et al. 1992; Stary, Chandler et al. 1994; Stary, Chandler et al. 1995).

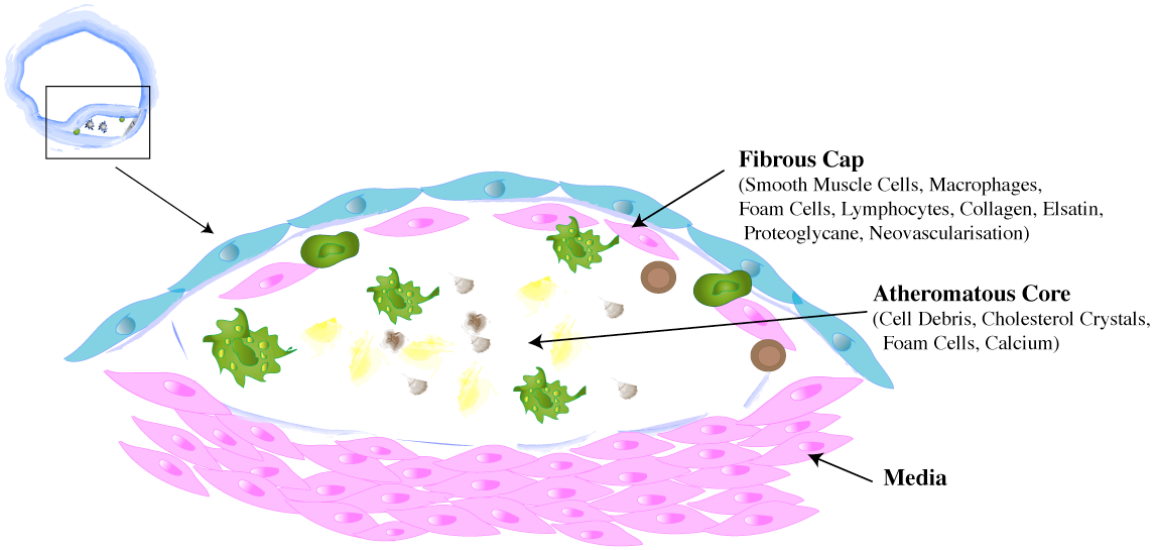

Figure 2. Major Components of a well-developed atherosclerotic plague

The hypotheses of atherogenesis

Atherosclerosis, reaching pandemic global distribution and affecting nearly every single one of us, prompted an explainable interest of researchers worldwide with ongoing intensive efforts trying to understand it's multifactorial pathogenesis. Numerous paradigms have been dominating opinions of the atherosclerotic community in this regard, some have been shown to be simply false such as a monoclonal smooth muscle cell proliferation hypothesis or a lipid accumulation theory, precluding passive intravascular lipid deposition and causing a degenerative slowly progressive disease of aging population. However, the most widely currently accepted view is that of atherosclerosis being a chronic inflammatory disease (Ross 1999), where the endothelial injury and response to it are postulated to be the driving forces behind the atheropathogenesis (Figure 3).

Ironically, in science as in life, most truths are not new. If we were to return to the 1850's Berlin, the "response to injury" hypothesis was in fact already than entertained by one of the most influential medical scientists of all times, Rudolf von Virchow. He argued that atheroma, the pathognomonic atherosclerotic lesion, is a product of inflammatory process within the intima and atheroslclerosis is a reaction to injury and inflammation within the arterial wall (Virchow 1856).

The modern formally prevailing concept of atherogenesis (Ross 1999) re-defines the "old truth" and adds "modernized" puzzle pieces, especially those of involved immunological and autoimmune components (Wick, Perschinka et al. 2001), to this certainly still scientifically challenging, a very complex multifactorial process.

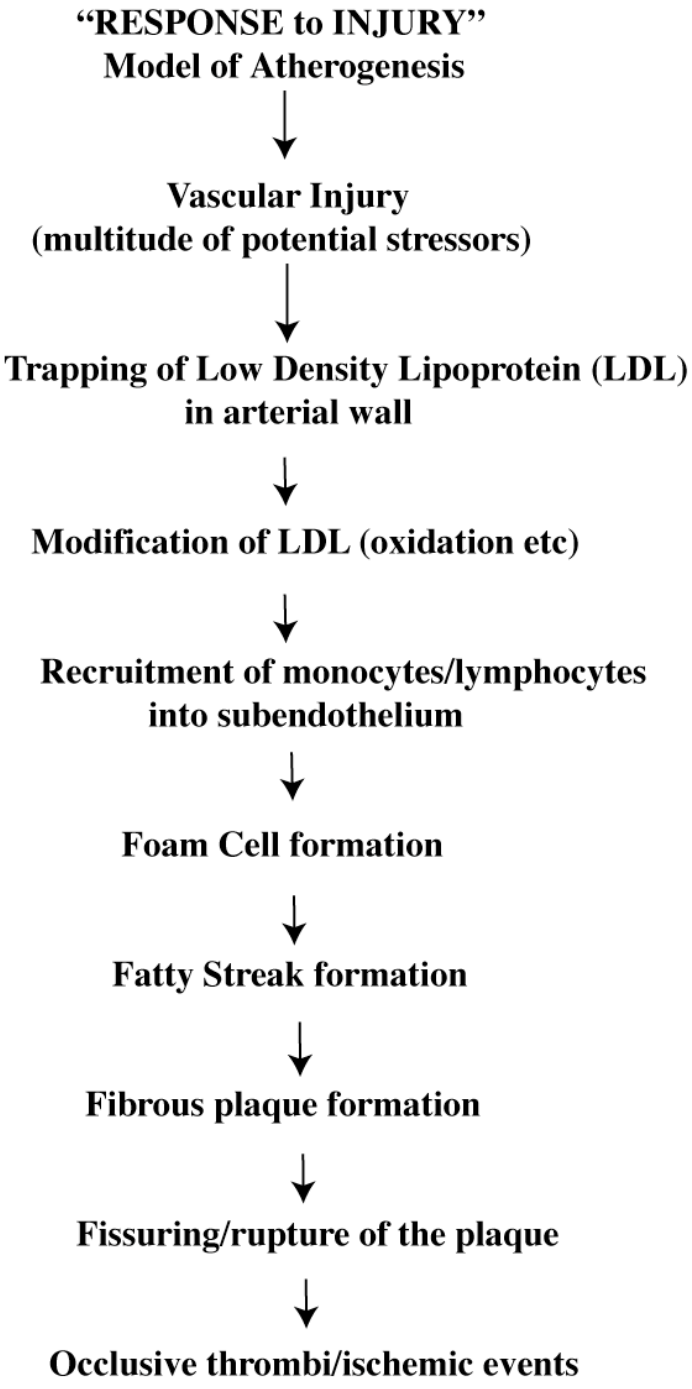

Figure 3. A "Response to Injury" Model of Atherogenesis
Adapted from Ross, R (1999)

Immune system and atherosclerosis

The immune competent cells are found to infiltrate the intimal spaces, are activated (Hansson, Holm et al. 1989) and secrete an array of pro-inflammatory cytokines (Frostegard, Ulfgren et al. 1999). Current hypothesis of atherogenesis prescribes the effector cellular roles in initiation, progression and culmination of atherosclerotic cardiovascular disease mainly to endothelium, macrophages and T-cells (Hansson 2005).

Among immunocompetent cells implicated in pathogenesis of atherosclerosis, dendritic cells (DC), mast cells, polymorphonuclear neutrophils (PMN), natural killer cells (NK) and B-cells have not been studied with the same passion as the other key players, such as macrophages and T-cells. A number of recent contributions are likely to illicit growing attention to these "partners in crime". Dendritic cells were identified within a vascular wall (Bobryshev and Lord 1995), a concept of the "vascular associated lymphoid tissue" (VALT) (Wick, Romen et al. 1997) analogous to the mucosal-associated lymphoid tissue has been coined. Mast cells (Kovanen, Kaartinen et al. 1995), NK (Linton, Major et al. 2004) remain marginalized, but claiming their right within the assembly and are subscribed a pro-atherogenic role (Getz 2005), while neutrophils could be expected to play a role during acute destabilization of the plaque (Gach, Nys et al. 2005).

B-cells are not found commonly within the plaque (Jonasson, Holm et al. 1986), nonetheless humoral responses are increasingly recognized as mediators of atherogenesis/atherothrombosis (Shoenfeld, Sherer et al. 2000), not least for their potentially protective function. Certain natural autoantibodies, recognizing phosphorylcholine (PC), present on the oxidized phospholipids. apoptotic cells and bactererial walls (for ex. of *Str.pneumoniae*) (Binder and Silverman 2005) interfering with oxLDL uptake by macrophages. These anti-PC antibodies have been proposed to play atheroprotective role in humans (Su, Georgiades et al. 2005).

Several candidate antigens have been implicated in the atherosclerosis, including modified low-density lipoproteins (oxidized; ox-LDL), heat-shock proteins (HSP), ß2-glycoprotein I (ß2-GPI) and infectious agents (Wick, Perschinka et al. 2001).

The importance of the immune system and specific immune reactions in atherosclerosis has been highlighted by recent interesting experiments, where atheroslclerosis in animal models was modulated by immunization procedures.

The role of immune reactions against oxLDL have been a matter of much debate. OxLDL can activate T-cells and antibodies against oxLDL are common in general population. Recent evidence indicates that increased anti-ox-LDL levels are associated with a decreased risk of atherosclerosis development, and immunization with oxLDL in an animal model leads to decrease of atherosclerotic lesions (Hansson 2005).

Heat shock (HSP) or stress proteins are highly conserved molecules with a range of functions including cyto-protection and the intracellular assembly, stabilization, folding and translocation of oligomeric proteins. Stress proteins are present in all species, and are induced by cellular insults including oxidative and haemodynamic stress, and inflammatory cytokines, all of which are associated with the development of cardiovascular disease. Heat shock proteins and antibodies against HSP have been hypothesized to play a role in initiating and promoting the immune reaction in the artery wall typical of atherosclerosis. According to this hypothesis, HSP functions as an innate danger signal, indicating for example that endothelial cells are stressed, which could be caused by oxLDL, hypertension and other stressors. HSP 60/65 and 70 elicit an immune response in normal healthy individuals, and it is therefore possible that stressed endothelial and other cells could be recognized by antibodies against HSP, and elicit an inflammatory reaction within the arterial wall (Wick, Perschinka et al. 2001).

In support of this hypothesis are a number of experimental studies, using animal models, and or observations in human subjects. Immunization with HSP 65 causes increased atherosclerosis in an animal model of atherosclerosis (Xu, Dietrich et al. 1992), and antibodies against HSP 60/65 have been associated with early atherosclerosis and cardiovascular disease in several studies (Xu, Willeit et al. 1993; Frostegard, Lemne et al. 1997). OxLDL per se has the capacity to induce HSP60/65 (Frostegard, Kjellman et al. 1996).

The natural history of atherosclerosis

Atherosclerosis is a slowly gradually progressive chronic inflammatory disease that begins in childhood, although we begin to appreciate that the lesions as well as progressing, can also regress during the lifetime. Occurrence of earliest morphologically evident atherosclerotic lesions, fatty streaks in aorta, have been shown by Holman et al to be present as early as in the first decade of life (Holman, Mc et al. 1958). These findings have been confirmed in the unique collection of vessels collected from 15-19 years of age at autopsy used for the Pathobiological Determinants of Atherosclerosis in Youth study (PDAY) by Strong et al (Strong, Malcom et al. 1999) reporting 60% of investigated aortae with cholesterol deposits and 60% of fatty streaks found in right coronary artery. The Holman study showed coronary artery affected already in the 2nd decade with more advanced lesions, fibrous plaques, present in coronary arteries in 3rd decade. Adult aorta may contain concomitantly plaques of any type and size from 0.3 to 1.5 cm in diameter at any developmental stage, from fatty streak to advanced coalesced calcified atheromas. Indicating that initiation of new lesions is not limited to an early age, but in fact can occur throughout adult life. This highlights the significance of our efforts in understanding the exact molecular mechanisms behind the initiating events.

The concept of " vulnerable" plaque

Predictive value of the plaque morphology and risk assessment has been of less than expected value, since many CVD events with lethal outcome were not occurring in patients within either "the high risk" zone based on the Framingham risk factors or having large plaques. In attempt to account for this understanding, a concept of the so-called "vulnerable" plaque, based on the biology of the culprit lesion, was recently introduced (Aikawa and Libby 2004). With mechanisms behind acute CVD being a formation of superimposed thrombus over a "vulnerable/active" plaque (Libby 2001). It is unclear what triggers the burst of inflammatory activity within a chronic lesion, however the evidence accumulating in favor of specific local antigen-driven response (Caligiuri, Paulsson et al. 2000; De Palma, Del Galdo et al. 2006).

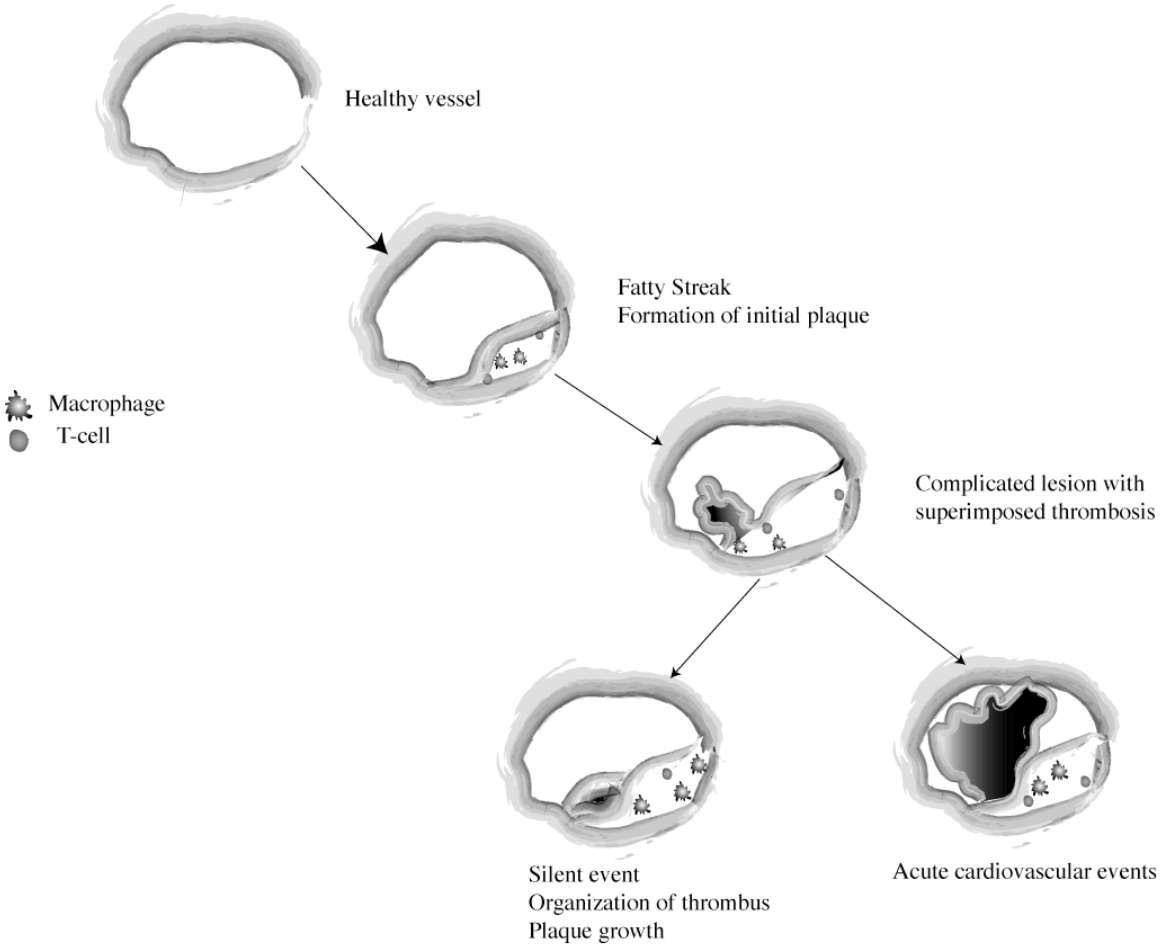

Figure 4. The evolution of an atherosclerotic lesion
Modified from P. Libby in Harrisson's Principles of Internal Medicine

The thrombogenicity of the atherosclerotic plaque

The thrombogenicity of an atherosclerotic lesion, whichever gross morphology it displays, is determined by the net effect of pro- and anti-coagulant factors *in situ* and in systemic surrounding milieu. The question of plaque thrombogenicity has been addressed in a large body of experimental work, concentrating initially on more dramatic alterations within the plaque architecture, such as fracture of the fibrous cap, leading to exposure of extracellular matrix, most notably of collagen, and sub-endothelial tissue factor (TF) to the flowing blood and triggering of immediate clot formation. More commonly events, triggering clotting in association with plaque, are of rather subtle nature (Libby 2001) for example superficial endothelial erosion, due to endothelial apoptosis or possibly

neovascular microthrombosis. Even though triggering of coagulation response might remain clinically silent most of the time, nevertheless, it reflects one of the mechanisms of plaque growth, namely by the intramural organization of the thrombus.

Among the procoagulant factors, cellular surfaces with exposed negative phospholipids, such as phosphatidylserine (PS) (Ananyeva, Kouiavskaia et al. 2002) and the presence of a membrane-bound glycoprotein, tissue factor (TF) (Toschi, Gallo et al. 1997) have major impact on the fate of the plaque. Anionic phospholipids exposed on activated or apoptotic cellular surfaces are essential in formation of both extrinsic and intrinsic procoagulant complexes, whereas TF is crucially responsible for activation of extrinsic blood coagulation pathway. Tissue factor expression is enhanced in the course of atherosclerosis (Wilcox, Smith et al. 1989), and is detectable on the foam cells, smooth muscle cells, endothelial cells and, in abundancy, within necrotic cores of plaques (Thiruvikraman, Guha et al. 1996), largely derived from the debris and microparticles released during apoptotic death (Mallat, Benamer et al. 2000). Expression of TF in close association with apoptosis (Hutter, Valdiviezo et al. 2004) is not surprising within the lipid-rich plaques, as activity and de-cryption of TF is, at least partly, co-dependent on PS expression (Wolberg, Monroe et al. 1999)

Risk factors for cardiovascular disease

In a claim to "conquest the coronary artery disease" (Kannel, Castelli et al. 1971), as rhetorically expressed by some involved investigators, an impressive epidemiological study was designed to follow up an entire community of a small US town for almost 40 years, chosen to represent and reflect the genetic composition and life style of the US nation. The city of Framingham became a very well known town to all cardiovascular researchers. Such reports from large epidemiological studies performed in North America and Europe have contributed to identification of numerous, now regarded as "traditional", risk factors associated with development and progression of atherosclerosis and CVD (Table 4). The evidence linking these factors with atherosclerosis is not only epidemiological but it is strengthened in experimental studies as well, including animal and *in vitro* findings (Glass and Witztum 2001).

Some of the traditional CVD risk factors clearly can and should be modified (and potentially reversed) such as smoking and obesity, by encouraging weight loss and increasing physical activity, while others such as male gender and increasing age are certainly there to stay and should be factitiously embraced and enjoyed.

Table 4. Traditional Risk Factors associated with Atherosclerosis
Hypercholesterolemia
Cigarette Smoking
Hypertension
Age
Male Gender
Family History
Diabetes Mellitus

The extent to which the established traditional factors explain the risk of CVD has been a question of debate, as their prevalence does not seem fully explain for the cardiovascular disease neither in general (Greenland, Knoll et al. 2003; Khot, Khot et al. 2003) nor in SLE population (Esdaile, Abrahamowicz et al. 2001). In search for additional independent promoters of CVD, observations that immune/inflammatory factors (novel factors) might play a role have emerged, including C-reactive protein (CRP) (Ridker 2003), homocysteine (Clarke, Daly et al. 1991), HSP-related factors (Xu, Willeit et al. 1993), aPLs (Hamsten, Norberg et al. 1986) and platelet activating factor-acetylhydrolase (PAF-AH) (Goudevenos, Tselepis et al. 2001) just to name a few. Importantly, the role of some of these factors is potentially beyond being simple biomarkers since their pro-atherogenic (for ex. CRP, PAF-AH) or atherothrombotic (for ex. aPLs) properties have been demonstrated.

Cardiovascular disease in systemic lupus erythematosus

Premature cardiovascular disease in systemic lupus erythematosus

The fascinating and real link between an exceedingly high incidence of premature cardiovascular disease and systemic autoimmune conditions, such as SLE, APS, RA, Wegeners granulomatosis (Frostegard 2005; Shoenfeld, Gerli et al. 2005), has been a subject of intense research in the last decade and has become a science of its own.

Epidemiological observations of CVD on the background of SLE have progressed since 1975 when unexpectedly advanced atherosclerotic lesions were found in young SLE patients in a widely cited autopsy series (Bulkley and Roberts 1975) and a clinical case presented by Urowitz et *al* in 1976, of a young 30 year-old female with acute myocardial infarction after 10 years of SLE. At the same time, bimodal pattern of mortality in SLE has been described, with the first peak occurring in early course of SLE due to infections or active disease and the second mortality peak, later in the course of disease, mostly due to CVD (Urowitz, Bookman et al. 1976).

The exceedingly high prevalence of premature cardiovascular disease in patients with SLE has been consistently demonstrated in epidemiological studies in all large well-characterized SLE-cohorts followed worldwide (Petri, Perez-Gutthann et al. 1992; Manzi, Meilahn et al. 1997; Manzi, Selzer et al. 1999; Svenungsson, Jensen-Urstad et al. 2001; Bjornadal, Yin et al. 2004), with a striking age-specific up to 50-fold increase of MI risk (Manzi, Meilahn et al. 1997).

Currently, SLE is diagnosed more often (Stahl-Hallengren, Jonsen et al. 2000) with a milder condition being recognized, and a better controlled disease activity, the CVD-associated mortality is the main cause behind the lower life-expectancy in SLE patients and therefore a serious clinical issue. However, it has yet failed to become a widely accepted knowledge, the problem remains underappreciated as demonstrated in a recent case presented by Rhew et al (Rhew and Ramsey-Goldman 2006), depicting the reality of young SLE patients. When a young 26 yrs-old lady, with SLE, was admitted with "textbook" symptoms pointing at possible MI, unfortunately MI was not included as

differential diagnostic consideration and she was home-discharged only to be re-admitted within 2 days with an extensive MI.

It has become customary to refer to the involvement of cardiovascular system in SLE as "accelerated atherosclerosis". Undoubtedly, atherosclerosis is an important underlying cause for manifested CVD in general population as well as in patients with systemic autoimmune diseases, however one should not discount vasculitis (Wurzel and Goldman 2004), possibly spastic phenomena and, even more importantly, increased propensity to thrombosis in these patients, as indicated by the CVD events in our cohort (**Papers I, III, IV**), where the majority of accounted events are of atherothrombotic nature. I would strongly argue that the term "accelerated atherosclerosis" does not truly reflect the entire phenomenon and the term " premature cardiovascular disease" would serve a better purpose

I believe there is a lack of discussion regarding another essential component in SLE-related CVD, that is of the fact that we are looking at affected patients who are predominantly women. Women are mysteriously different or rather not (to some), physiologically complicated and immunologically different creatures. Compared to male vasculature, female vessels are smaller, independent of body size, (Pepine, Kerensky et al. 2006) and are different in that they are under prolonged cycle-like influence of estrogens during reproductive years. Generally, women are protected from CVD until the postmenopausal time but in SLE the risk for CVD is exceedingly high particularly in young pre-menopausal females (Manzi, Meilahn et al. 1997). When presented into emergency, women are given less consideration regarding possible MI, the symptomatology is less clear than in the case with male patients, a less aggressive treatment is administered and female CVD mortality is higher (Tunstall-Pedoe, Kuulasmaa et al. 1999).

Mechanisms of cardiovascular disease in SLE

The contributive mechanisms of pathogenesis of CVD in SLE are multiple and incompletely understood (Manzi and Wasko 2001; Frostegard 2005). Many critical questions remain unanswered. Since prevalence of traditional risk factors alone does not explain the link CVD-SLE (Esdaile, Abrahamowicz et al. 2001), it is essential to

understand if there are other, novel or lupus-specific factors, directly contributing to premature CVD? If so, which factors/mechanisms are predominantly pro-atherogenic/atherothrombotic and which (if any) are able to confer protection against CVD? Is the effect of traditional risk factors different on the background of systemic inflammatory conditions? And what are the interrelationships between traditional and lupus-specific inflammation/treatment-related factors (synergistic, additive)? Is there only a subgroup of patients prone to premature CVD, within those who satisfy the criteria for SLE, since SLE is a clinical definition for heterogeneous number of syndromes? Is there a unique combination of factors in a subset of SLE patients?

These questions are not of pure research interest but are clinically important. Identification of SLE patients, who are at higher risk for CVD and deserve aggressive treatment/control of known risk factors, will so importantly protect these patients from additional burden of CVD-related morbidity and prevent untimely and unnecessary deaths.

Does atherosclerosis matter or is it more atherothrombosis?

The related literature almost uniformly states "accelerated atherosclerosis" in SLE as the cause behind premature CVD events. The true frequency of atherosclerosis is difficult to determine accurately because of its asymptomatic presentation. Using the surrogate measurements to assess atherosclerotic disease in its sub-clinical stage, a intimal-media thickness (IMT) of the vessel can be measured in the sites prone to atherosclerosis such as carotid artery and femoral arteries by a relatively simple US Doppler technique**.** Modern techniques also allow even more elaborate determinations such as visualization of intra-coronary calcifications a hallmark of advanced plaques by spiral computer tomography. Available epidemiological evidence highlights earlier atherosclerotic development in SLE patients, as determined by thickening of vascular wall, presence of plaque (Svenungsson, Jensen-Urstad et al. 2001; Roman, Shanker et al. 2003) as well as vascular stiffening (Selzer, Sutton-Tyrrell et al. 2001) as compared to their age-matched healthy counterparts. Overall, studies seem to find prevalence of atherosclerosis 31-37 % in SLE patients as compared with 9 to 15 % of controls (Hahn 2003), more striking and apparent is the difference in SLE patients over 50 years, where 70-80% were positive at

least by some surrogate measure of atherosclerosis compared with 21-45 % of age and race-matched controls. Hence, the evidence seem to support the notion that SLE patients have prematurely increased burden of atherosclerosis ("accelerated atherosclerosis").

At the same time, commonly used relevant endpoints of clinically manifested CVD are objectively evidenced events of MI, ischemic stroke, as well as angina pectoris and claudications. And again, repetitively, the risk for actual CVD events is exceedingly high (up to 50 fold) particularly in young pre-menopausal females (between 35-44 yrs) (Manzi, Meilahn et al. 1997). Acute cardiovascular events are a result of complex interaction between the thrombogenicity of the plaque, state of activation and inhibition of the coagulation cascade as well as fibrinolytic effects and the vasomotor function (Libby 2001). In SLE, the vulnerability of atherosclerotic plaque, its thrombogenicity and predisposition to rupture might be of uttermost importance.

Therefore the posed question "does atherosclerosis matter or is it more atherothrombosis?" should be answered as "potentially both". One could envision SLE-related CVD being caused by a detrimental combination of increased and earlier burden atherosclerosis (both plaques and increased vascular stiffness) and an enhanced propensity to thrombosis (Figure 5).

Risk factors for premature cardiovascular disease in SLE

Certainly, age and other traditional risk factors (Table 4) play a role in SLE (Petri, Spence et al. 1992; Manzi, Selzer et al. 1999). Sedentary life style (related to fatigue, joint and muscle pain experienced by most patients), obesity and hypertension often escape attention of physicians, fearing additional medication load to already "polypharmacized" SLE-patients and traditional CVD risk factors are not treated aggressively (Hahn 2003). Albeit, the prevalence of traditional risk factors alone can not account for the incidence of CVD in SLE and presence of SLE *per se* is found to be independently associated with CVD (Esdaile, Abrahamowicz et al. 2001).

In recent studies from our group, several traditional and non-traditional risk factors in relation to CVD were studied and reported by E. Svenungsson in her thesis (Svenungsson, Jensen-Urstad et al. 2001; Svenungsson 2003). Briefly, markers of systemic inflammation (reflected by raised levels of acute phase reactants and TNF-α),

dyslipidemia (raised triglycerides (TG) and low high-density lipoprotein (HDL)), increased levels of homocysteine, enhanced oxidation of LDL and antiphospholipid antibodies (lupus anticoagulant and anti-oxLDL antibodies) were characteristic of women with SLE and a history of CVD (Svenungsson 2003; Svenungsson, Fei et al. 2003; Frostegard, Svenungsson et al. 2005).

Are there parallels between the inflammatory mechanisms in CVD and SLE?

Yes, there certainly are (Kao, Sabatine et al. 2003). One also might wonder if SLE and atherosclerosis help open "the door of autoimmunity" for each other. As demonstrated by clinical associations between for example SLE and other autoimmune diseases, such as systemic sclerosis, myasthenia gravis (Amital 1999). Systemic inflammatory condition with an autoimmune component might accelerate atherogenesis or promote thrombosis by a number of mechanisms (Frostegard 2005; Shoenfeld, Gerli et al. 2005).

Apart from generally sharing similar effector immune mechanisms (innate and adaptive immune components, also vascular injury, leading to activation and consequent dysfunction of endothelium (Vallance, Collier et al. 1997; Hunt and Jurd 1998) as well as EC apoptosis are common features of both SLE and atherosclerosis (Salmon and Gordon 1999; Chang, Binder et al. 2004). In the setting of lupus, oxidative stress is higher, our group demonstrated increased lipid peroxidation (Frostegard, Svenungsson et al. 2005). The complement system has been implicated in both conditions (Seifert and Kazatchkine 1988; Manderson, Botto et al. 2004). The course of SLE is characterized by periods of flares followed by the relatively calm periods of remission, which is perhaps similar in case of atherosclerosis.

Microorganisms have been implicated as a potential ethiologic factor associated with SLE, where in particular Epstein-Barr virus, trophic for B-cells (McClain, Heinlen et al. 2005) is a focus. Recently, EBV DNA and EBV responsive T-cells were demonstrated in unstable atherosclerotic lesions (de Boer, Teeling et al. 2006). Accumulating evidence suggests that infection, in combination with other pro-atherogenic factors, is most likely to participate in promoting atherosclerosis (Katz and Shannon 2006). Epidemiological observations support association of *Chlamydia pneumoniae* (Saikku, Leinonen et al. 1988), periodontal bacteriae (Buhlin, Gustafsson et al. 2003) with cardiovascular disease.

While a direct causal link between any particular infectious agent and initiation of atherosclerosis has not been proven, detection of colonization of advanced atherosclerotic plaques by diverse microorganisms and not in early lesions (Ott, El Mokhtari et al. 2006) supports the possible acceleration of atherosclerosis by microorganisms.

Treatment-related factors in SLE, such as corticosteroids, a commonplace therapeutic option, might contribute to progression of plaque in a number of ways (Hahn 2003). At the same time Roman et al (Roman, Shanker et al. 2003) reported an interesting association, where patients without plaques, seemed to have been treated more aggressively with corticosteroids as well as they received hydroxychloroquine and cyclophosphamide more frequently. Speculatively, it might be an indirect confirmation that immunosupression could prevent progression of atherosclerosis (even in general population?). Interestingly, hydroxychloroquine, an antimalarial agent, was shown to have a weak but real lipid-lowering effect of its own (Rahman, Gladman et al. 1999).

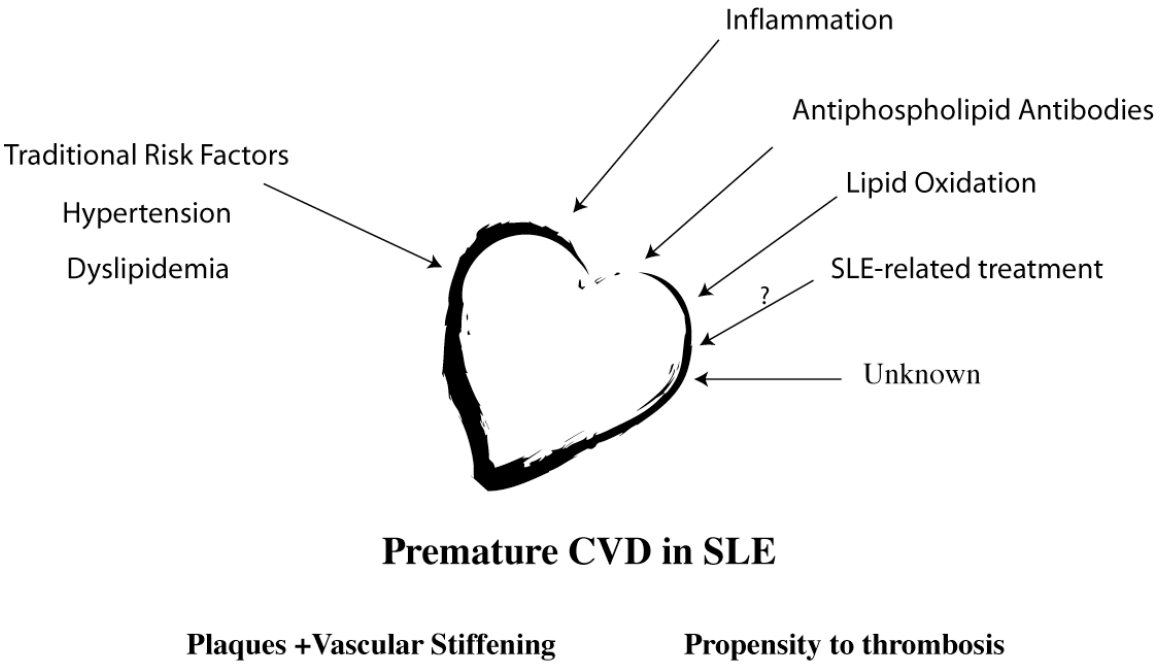

Figure 5. Premature cardiovascular disease in SLE

Modified from J. Frostegård 2005

The Endothelium

Endothelial cells (EC) form a continuous single-cell lining sheet over the entire vascular system, collectively designated as endothelium. An average 70 kg adult human body contains 2 kg of endothelium in weight and covers impressive 6.5 tennis courts in surface size. Endothelium is indeed one of the largest organs, located at a strategically important blood/tissues interface. A historical notion dominating vascular biology, portraying endothelium as a passive barrier, simply dividing circulating blood from the tissues, has been radically changed with the rightful appreciation of highly active and dynamic character of this barrier. Indeed, under physiological conditions, while performing functions of a semi-permeable filter, endothelium maintains a non-thrombogenic surface and rapidly responds to the requirements of vasomotor control, both functions being crucial for healthy circulatory flow. In summary, integrity of endothelium, both structural and functional, is essential for maintenance of vascular and circulatory homeostasis (Houston 2002).

Table 5. Regulatory Functions of Endothelium
Vasomotor Tone
Hemostasis and Thrombosis
Selective Permeability Barrier
Immune and Inflammatory Reactions
Vascular Growth and Remodeling

The properties of healthy endothelium are in principle thromboresistant. It is achieved by combining anticoagulant, fibrinolytic and anti-platelet aggregation mechanisms. Normal physiologic endothelial phenotype is not able to support pro-coagulant reactions, there is no overt expression of initiators of coagulation cascade (Houston 2002).

Table 6. Anti-and pro-thrombotic endothelial mediators	
Anti-thrombotic	***Pro-thrombotic***
Thrombomodulin (TM)	Thromboxane A2 (TXA2)
Nitric Oxide (NO)	Tissue Factor (TF)
Heparin-like proteoglycanes	Plasminogen Activator Inhibitor-1 (PAI-1)
Tissue Plasminogen Activator (tPA)	Von Willebrand Factor (vWF)
Prostacyclin (PGI2)	Platelet- Activating Factor (PAF)

Modified from M. Houston (Houston 2002)

The lining monolayer is formed by a structurally heterogeneous endothelium in different anatomical locations (high venules, arteries, veins), reflecting different functional requirements. Physiologic arterial hemodynamic shear stress is clearly different from that of dominating in the low-pressure venous system and potentially being of importance for development of disease and may influence responsiveness to triggers of atherosclerosis (Davies, Spaan et al. 2005)

It is of uttermost importance to keep in mind the complexity and integral interdependency of pro- and contra acting mechanisms on the molecular levels. If we were to ignore such physiological unity, we are bound to be painfully reminded about it at high costs. Similar to the scale of the recent events associated with a launch of selective cyclooxygenase-2 (COX-2) inhibitors, that apart from offering certain anti-inflammatory benefits, are able to kill. Speculatively by shifting the fine balance between prostacycline (PGE_2) and thromboxane A_2 (TXA_2) in the vasculature and depriving the patient from efficient regulatory mechanisms, preventing CVD events. Another possibility is that the introduction of selective COX-2 inhibition in the group of patients with RA, predominantly being prescribed this class of drugs, shifts the already dysfunctional endothelial protective mechanisms into even more destabilized status (Hochberg 2005).

Endothelium in cardiovascular disease

The endothelium is primarily affected during inflammation and is an important player in pathologic vascular processes such as atherosclerosis and atherothrombosis. An endothelial cell is the first to be exposed to/ affected by and suffer from stressful influences of various traditional and non-traditional risk factors (blood-borne substances

and hemodynamic influences) associated with cardiovascular disease. Pathophysiological stimuli such as proinflammatory cytokines, advanced glycation end products (AGES), chronic exposures to hypercholesterolemia or hyperhomocysteinemia among others are believed to be critical in inflicting endothelial injury leading to initiation and progression of atherosclerosis. However the endothelium is only the first cell to be influenced, while the vessel is affected as a whole structural unit, with smooth muscle cells, extracellular matrix and adventitia dragged to participate in the pathological process.

Multiple factors potentially able to modulate endothelial phenotype, changing it to activated and dysfunctional, are found to be increased in patients with SLE (Frostegard 2005). One might envision SLE patients as having a state of generalized "poor endothelial health".

Such understanding makes endothelium a target of interest for primary prevention and endothelial health the ultimate goal in treatment of atherosclerosis. Since the endothelial injury/dysfunction precedes manifested CVD by years, availability of a suitable marker, ideally with an independent predictive value and specific enough to be used in epidemiological studies, would allow identification of high-risk individuals in whom more aggressive prevention guidelines could be warranted.

Soluble vascular cellular adhesion molecule-1 (sVCAM-1)

Vascular cellular adhesion molecule-1 (VCAM-1) is a subject of special interest in atherosclerosis research, since this particular CAM, expressed on activated endothelium, is crucial in the process of firm adhesion of monocytes and lymphocytes (Cybulsky and Gimbrone 1991). The interaction between VCAM-1 and a very late activation-antigen 4 (VLA-4) is a prerequisite to the recruitment and subsequent transmigration of monocytes and T-cells, both key cellular players in atherogenesis (Hansson 2005), to the subendothelium (Figure 6). Recently, a preferential expression of VCAM-1 in response to hemodynamic stress was demonstrated in endothelium derived from atherosclerosis-prone sites (Dai, Kaazempur-Mofrad et al. 2004). In humans the issue of cleavage of VCAM-1 from the endothelial surface has not been resolved as well as a potential functional activity of sVCAM-1 versus simply being a biomarker, reflecting the state of endothelial activation.

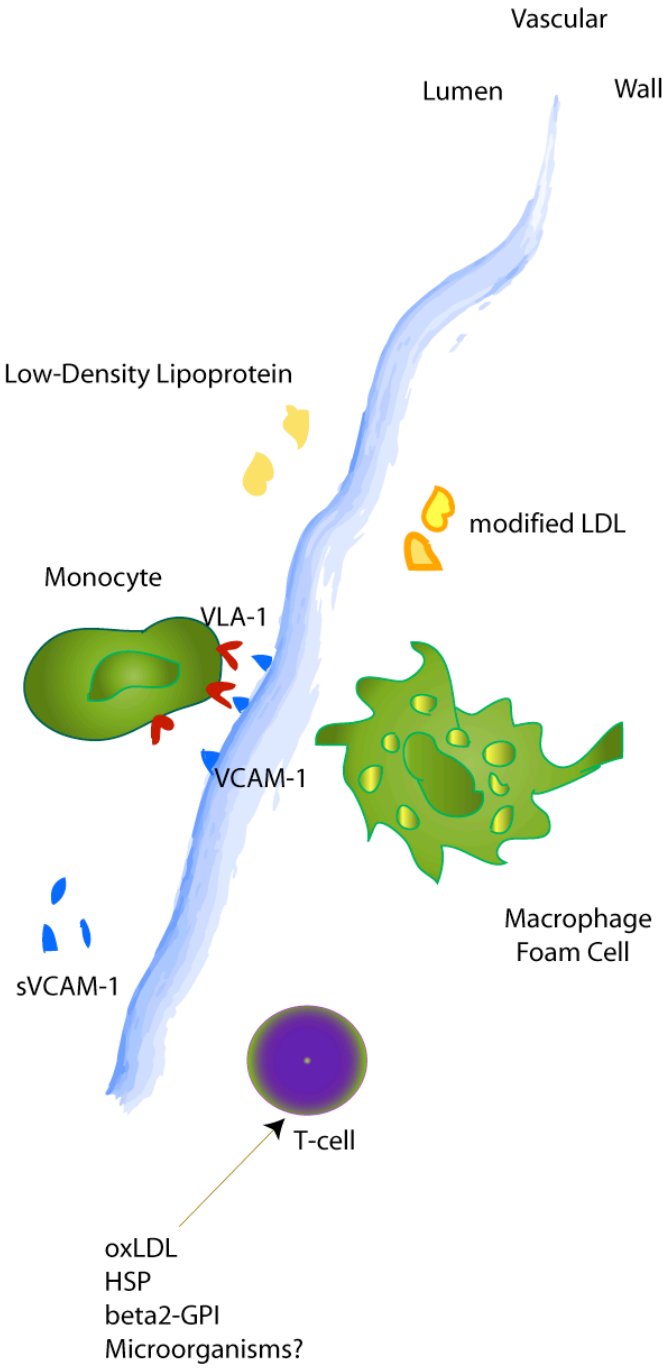

Figure 6. Vascular Cellular Adhesion Molecule-1 in atherogenesis

Soluble Thrombomodulin (sTM)

Thrombomodulin (TM) is glycoprotein, a constitutive member of EC surface repertoire, acts as an anticoagulant, mainly by its extra-cellular domain serving as a high-affinity

receptor to thrombin (Esmon and Owen 2004). Ishii et al reported TM to be increased after EC damage in cultures (Ishii, Uchiyama et al. 1991). Significantly increased levels of sTM were reported in SLE patients with aPL and attributed to potential aPL-induced damage by Karamochkine et al (Karmochkine, Boffa et al. 1992). Heterogenous soluble circulating TM fragments were shown to retain significant cofactor activity (up to 30-50 % of that surface expressed TM) (Uehara, Gotoh et al. 2001). In general population, patients with increased levels of sTM has been demonstrated to do worse in respect to mortality and reinfarction after acute coronary syndromes (Chan, Chen et al. 2005).

Materials and methods

Patients and Study Design (Papers I, III, IV)

The patients who fulfilled four or more ACR criteria (Tan, Cohen et al. 1982) for SLE were asked to participate in the studies, 208 have agreed and were included in the SLE cohort in the period between 1995-1999 at the Karolinska Hospital. For each patient, investigations included a routine evaluation by a rheumatologist, an interview and a blood sampling after an overnight fasting.

A nested case-control study was designed (Figure 7), when out of 208 patients, all but one women (n=26) surviving one or more CVD events were included (SLE cases). CVD events were defined as myocardial infarction (confirmed by ECG and creatine kinase; n=7); angina pectoris (confirmed by exercise stress test; n=9); cerebrovascular events (ischemic but not vasculitic or haemorrhagic stroke, confirmed by CT or MRI; n=15); and claudications (peripheral vascular disease; confirmed by angiography; n=4).

The SLE controls were chosen out of 208 SLE patients, based on their negative history of CVD events as defined above and age-match (within 6 months) to the SLE cases. Age-matched population controls were recruited from the Swedish population registry.

The studies were approved by the local Ethics Committee of the Karolinska Hospital and all subjects gave their informed consent.

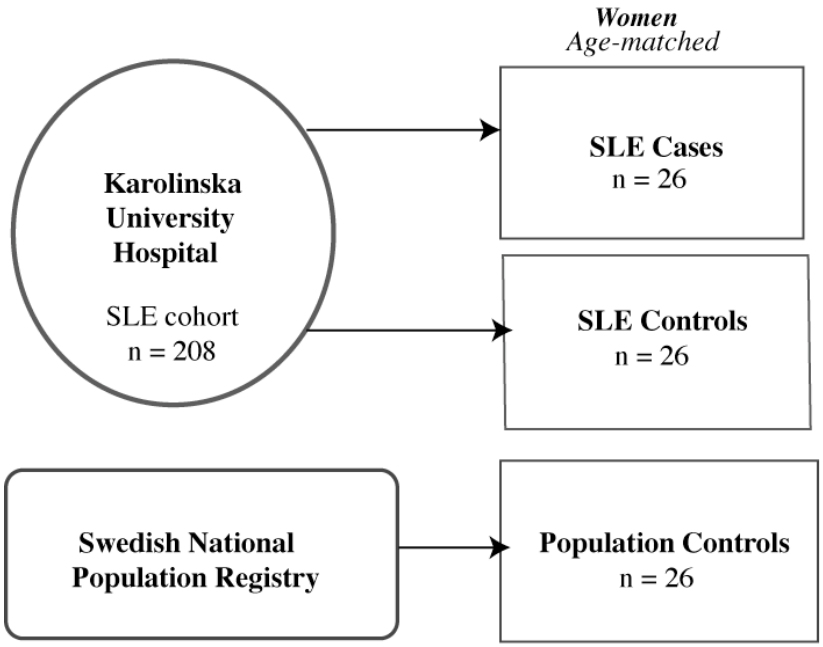

Figure 7. Nested case-control study design

Human Umbilical Venous Endothelial Cells (Papers I, II, III)

Pooled HUVECs at passage 2 were purchased from a commercial source (from Cascade Biologics, Inc., Portland, Oregon, USA). The same batch of HUVECs at passages 3 to 5 was used throughout experimental work. HUVECs were maintained in basal endothelial growth medium (EGM) (Clonetics, San Diego, CA, USA), phenol red-free, containing 2% of fetal bovine serum and recombinant human epidermal growth factor (rhEGF), hydrocortisone, bovine brain extract (BBE), gentamicine-sulphate and amphotericin-B (GA-1000). Until 70-80% of confluence the cells were incubated in 75 cm^2 flasks (TPP, AG, Trasadingen, Switzerland) at 37ºC under humidified 5% CO_2 conditions.

Flow Cytometry (Papers I, II)

Flow cytometry on HUVECs was performed with the FACScan flow cytometer (BD Biosciences, San Jose, CA, USA). All data acquisition and analysis was done with CellQuest™ Software.

Annexin A5-binding assay

Assessment of ANXA5 binding to HUVECs was performed. Briefly, cells were seeded at $2x10^4$ cells/ml density in 12-well plates (NUNC, Inc, Naperville, IL, USA). After allowing 12-24 hours for attachment, HUVECs were kept in serum-free medium (SFM) for at least 12 hrs prior to treatment with various samples. Cells were harvested with Cell Dissociation Solution (CDS; Sigma-Aldrich, St. Louis, MO, USA) and carefully pooled with supernatants, followed by centrifugation at 1200 rpm for 7 min. After resuspension in 150μl of annexin V-binding buffer (10 mmol/l HEPES, 140 mmol/l NaCl and 2.5 mmol/l $CaCl_2$; Molecular Probes Inc, Eugene, OR, USA) samples were stained with 2μl of annexin V conjugated with fluorescein isothiocyanate (FITC) **(Paper I)** or with allophycocyanin (APC) **(Paper II)** (Mol.Probes, Inc.) and incubated for 15 min on ice. Shortly before acquisition 1μg/ml of propidium iodide (PI; Mol. Probes) was added.

Data acquisition was started within 1 hr after harvesting of HUVECs. A gate was set to exclude events smaller than 230 on linear FSC and SSC scales. For each sample 10000 events were collected within this gate, unless specified otherwise. HUVECs cultured under basal conditions (in complete medium) or subjected to 24 hrs serum starvation were prepared in quadriplicates as controls for each experiment. The frequency of $ANXA5^{pos}$ events was determined as percentage of $ANXA5^{pos}/PI^{neg}$ cells on a bivariate dot plot or as a percentage of $ANXA5^{pos}$ cells on a histogram **(Paper I).** Median fluorescence intensity (MFI) of ANXA5 binding to HUVECs **(Paper II)** was determined by setting a gate over $ANXA5^{pos}$ population on a histogram.

Chromatography (Paper I)

We have purified the IgG fraction from the pooled sera with high aPL titers using chromatography separation method with high-affinity Protein G column (HiTrap Protein G HP) from Amersham Biosciences AB (Uppsala, Sweden).

Immunohistochemical staining of human atherosclerotic plaques (Paper I)

Formaldehyde-fixed specimens of human atherosclerotic lesions were stained with monoclonal anti-annexin V antibody of mouse type IgG_{2a}(Alexis Biochemicals, Corp, Lausen, Switzerland) as described in Paper I.

Determination of anti-annexin A5 (Paper I) and anti-endothelial cell (Paper III) antibodies

Anti-Annexin A5 IgG antibodies were determined by Hemochrom Diagnostica ELISA kit (Essen, Germany). For determination of anti-endothelial cell antibodies an "in-house" ELISA was used as described.

Determinations of cell viability and apoptosis- related processes (Paper I)

The internucleosomal splicing of DNA

Accumulation of apoptosis-related internucleosomal DNA fragments in cytoplasma of HUVECs was determined by ELISA(Roche Diagnostics GmbH, Mannheim, Germany)

The MTT assay

The reduction of MTT [3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium-bromide] salt was determined with Vybrant™Cell proliferation Assay kit (Mol.Probes) according to manufacturer's instructions.

Flow cytometry

Permeability of cellular membrane was assessed with a vital dye propidium iodide (PI). Changes of cellular morphology (size and granularity) were assessed on forward-scatter (FCS) and side-scatter (SSC) parameters (data not shown).

Human monoclonal antibody against cardiolipin (aCL) (Paper II)

Human monoclonal antibody against cardiolipin (aCL) (HL5B) of IgG_2 subclass were generated with the polyethylene glycol (PEG) chemical fusion method. B-cells were derived from a patient with antiphospholipid syndrome, transformed by EBV and fused with a mouse-human heteromyeloma cell line essentially as described (Buschmann, Fischer et al. 2005). Prior experiments, aCL preparations were preserved without azid at -20°C.

Measurements of subclinical atherosclerosis (Papers I, III, IV)

The intima media thickness (IMT) was determined by in the right and left carotid arteries by the ultrasound Duplex scanner (Acuson Sequoia Mountain View, Ca). Plaque was defined as a local intimal/medial thickening, with a thickness greater than 1 mm and an increase (100%) in wall thickness compared to the adjacent wall segments. The score "absent vs present" was used.

Routine laboratory and cytokines measurements (Papers I, III, IV)

Routine clinical laboratory techniques were used to determine acute-phase reactants and blood lipoproteins. CRP was measured with high-sensitivity assay. aCL antibodies were measured by enzyme-linked immunosorbent assay (ELISA) with ethanol fixed cardiolipin (Sigma). Lupus anticoagulant (LAC) was determined by the modified Dilute Russel Viper Venome method, with Bioclot lupus anticoagulant (Biopool, Umea, Sweden). TNF-alpha and soluble TNF-receptors were measured by commercially available ELISA kits (R&DSystems, Minneapolis, MN, USA). These results have been reported earlier (Svenungsson 2003).

Levels of ANXA5 (**Paper I**) were determined by commercial high-sensitivity ELISA kit (Hemochrom Diagnostica GmbH, Essen, Germany), with the indicated detection range within 0.2-20 ng/ml.

PAF-AH activity measurements (Paper III)

Activity of PAF-AH in plasma was measured with the trichloracetic acid precipitation procedure (Goudevenos, Tselepis et al. 2001) and expressed in nanomoles of radioactively labeled substrate, acetyl-PAF-AH, hydrolyzed per minute per ml of plasma.

Measurements of HSP-related factors

Serum levels of HSP 60 and HSP 70 were determined by ELISA as described earlier (Pockley, Wu et al. 2000). Anti-HSP 60, anti-HSP 70 and anti-HSP 75 antibody levels were determined by ELISA as well (Pockley, Wu et al. 2000).

Assessment of flow and nitroglycerine–mediated vasodilatation (Paper IV)

The method described by Celermajer et al (Celermajer, Sorensen et al. 1992) was used for assessment of the vasodilatation (A.Brachialis) evoked by reactive hyperemia (flow-mediated dilatation; FMD) or by sublingual nitroglycerine administration (nitroglycerine-mediated dilatation; NMD). The changes of vessel diameter are expressed as percentages of the first (baseline) control scan. All ultrasound scans were made using a duplex scanner (Acuson Sequoia, Mountain View, California, USA).

Statistical methods

The statistics were computed using StatView software (SAS Institute AB, Göteborg, Sweden). Skewed continuous variable were logarithmically transformed. Study groups were compared using ANOVA for continuous variables, with Fisher's PDSL as post hoc analysis. Correlation coefficients were calculated using simple regression, or for not normally distributed variables, Spearman Rank correlation. The significance level was set at $p<0.05$.

Results and Discussion

Annexin A5 binding to endothelial cells

The frequency of HUVECs positive for ANXA5 was significantly lower when incubated with plasma of SLE cases as compared to SLE controls and population controls. An essential issue was to determine if the cells cultured with SLE serum were in fact affected in their viability and if any association between ANXA5 binding in our system was related to a possible induction of apoptosis.

Flow cytometric analysis on a single cell level (we collected 10 000 events within a "non-debri" gate/sample), allowed simultaneous evaluation of cellular morphology (FCS/SSC) and evaluated the integrity of plasma membrane with the vital dye propidium iodide (PI). We found no significant differences in either morphologic changes or frequency of $PI^{bright+}$ cells, indicating no cytotoxic effect of plasma on HUVECs (data not shown). At the same time there were no detectable differences in the ability of HUVECs to reduce the MTT component to its insoluble counterpart. Induction of apoptosis-specific DNA fragments was not different between the three groups. Even though cell death might not be exclusively accompanied by the "classical" features consistent with either apoptosis or necrosis, in our system with HUVECs we have validated methods such as DNA inter-nucleosomal splicing, MTT, flow cytometric analysis (ANXA5 binding, morphology, PI) with positive (serum-starvation induced apoptosis) and negative (basal culture conditions) (data not shown).

Anticardiolipin antibodies and Annexin A5 binding

In the **Paper II** we report that serum from individuals with high aPLs titers induce decreased ANXA5 binding to HUVECs as compared to the healthy controls. Likewise, when we used a monoclonal aCL-mAb derived from an APS patient (Buschmann, Fischer et al. 2005) we observed a decrease of ANXA5 binding to HUVECs. Please see paper II for discussion.

IVIG and Annexin A5 binding

In **Paper II** we report that preincubation of APS sera with IVIG at therapeutic levels restored ANXA5 binding comparable to that of healthy controls. Likewise, preincubation of IVIG with aCL-mAb restored ANXA5 binding to HUVECs.

Interestingly, added to in normal serum and used by itself, IVIG induced small but detectable decrease in ANXA5 binding. Please see paper II for discussion.

Annexin A5 in atherosclerotic plaques

In **Paper I** we demonstrate abundant presence of ANXA5 in all examined advanced atherosclerotic lesions (n=11) derived from endarterectomy of non-SLE patients in addition to one lesion from an SLE case, while no ANXA5 was detected in a morphologically healthy artery from mesenteric vasculature. ANXA5 was detected by immunohistochemistry consistently in sites with well-recognized high prothrombotic potential such as an atheromatous lipid-rich core, vasa vasorum within the shoulder regions. At the same time we have observed significant positive correlation between ANXA5 binding and IMT and plaque presence in SLE cases.

Our finding of intensive ANXA5 staining in vicinity of macrophages (as stained with macrophage-specific anti-CD68 marker) is in line with similar findings in inflamed synovial tissue from RA patients (L.Sehlstedt, 2004 unpublished). These are interesting findings deserving further exploration.

The best characterized, but perhaps not an exclusive, signal for phagocytic engulfment on apoptotic cellular membrane is phosphatidylserine (Fadok, Bratton et al. 1998). Affinity of ANXA5 to this particular phospholipid (Koopman, Reutelingsperger et al. 1994) may be a double-edge sword, while shielding procoagulant surfaces ANXA5 might inhibit the uptake of apoptotic cells as demonstrated *in vitro* if present around during the execution of apoptosis (Kenis, van Genderen et al. 2005), which not only could significantly impair clearance but also increase the immunogenic potential of apoptotic material as demonstrated in animal studies(Stach, Turnay et al. 2000).

We therefore propose that within the vessel wall ANXA5 might act primarily as a “band-aid” over activated endothelial surfaces (a layer-like deposition of ANXA5 was detected in luminal endothelial surfaces over the plaque) and at the same time contribute to an

increase of plaque size, by impeding clearance of apoptotic cells especially in progressed stages when the amount of apoptotic debris within atheroma is likely to increase.

Novel risk and protective factors for cardiovascular disease (Paper III)

We have compared emerging CVD risk factors including platelet activating factor-acetylhydrolase (PAF-AH) and soluble phospholipase A2 (PLA2); heat shock protein (HSP)-related measurements and antibodies against endothelial cells (aEC). Of all these measurements, only activity of the PAF-AH was significantly associated with CVD in SLE. We hypothesize that PAF-AH promotes inflammation and atherosclerosis in SLE. Please see paper III for discussion.

Biomarkers/measurements of endothelial status associated with cardiovascular disease (Paper IV)

In this study, we report that FMD values did not differ between SLE patients without CVD and population controls. High FMD values indicating good endothelial function were even more common in SLE. Soluble VCAM-1 discriminated between SLE cases vs SLE controls and population controls. Levels of soluble thrombomodulin were higher in both SLE groups than in population controls but differed only trendwise between SLE cases and SLE controls. Please see paper IV for discussion.

Concluding Remarks

In the course of these PhD studies I have been challenged repetitively to argument for the belief, that women with SLE represent indeed a "good " model for human cardiovascular disease. Although, animal models of atherosclerosis are essential in basic research focusing on delineating pathogenic mechanisms, murine atherosclerotic plaques display discrepancies in morphology compared to human lesions, and more importantly, they fail to develop spontaneous atherothrombosis even in a factitious presence of advanced plaques. By studying human subjects with SLE and CVD, we entertain the hope that by better understanding the interplay between multiple factors and mechanisms participating in the pathogenesis of atherosclerosis/cardiovascular disease in SLE, we could not only acquire knowledge exclusively of benefit to this specific subgroup of patients but a knowledge which could add to a better insight in these processes in general population. I am convinced that this well designed nested case-control study, focusing on SLE-related CVD, has contributed to a partial realization of such expectations.

In conclusion, a novel mechanisms in SLE-related CVD has been demonstrated, that may play an important role in promoting atherothrombosis, namely aPL-induced decreased binding of antithrombotic Annexin A5 to endothelium.

We hypothesize that increasing Annexin A5 binding could represent a novel therapy against atherothrombosis, either by administration of Annexin A5, or by neutralizing aPL when present, with neutralizing antibodies from IVIG. Of note, IVIG may also per se have prothrombotic effects through this mechanism.

PAF-acetylhydrolase activity and endothelial cell activation may also play a role in promoting CVD and atherosclerosis in SLE.

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