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IMMUNE MECHANISMS IN ATHEROSCLEROTIC VASCULAR DISEASE

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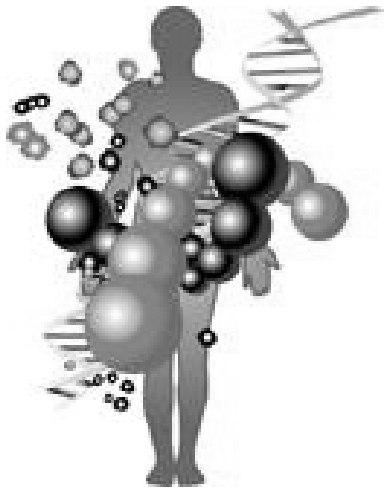

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"Omnium rerum principia parva sunt."

Marcus Tullius Cicero

To my mother Srbinka

"Everything has a small beginning."

Marcus Tullius Cicero

Abstract

Atherosclerosis has been categorized as an inflammatory disorder, with similar chronic inflammatory conditions found in rheumatoid arthritis. It entails an unregulated accumulation of lipids in the vessel wall, which leads to the development of cardiovascular diseases. The WHO report from 1999 showed that cardiovascular diseases are the major cause of mortality and morbidity in Westernized countries. Antigens found in atherosclerotic lesions, like oxidized LDL or cardiolipin, induce humoral immune factors, such as anticardiolipin antibodies (aCL), antibodies against oxidized forms of LDL, circulating immune complexes (CIC) and pro-inflammatory cytokines, IL-1β and TNFα.

Anticardiolipin antibodies have been considered as risk factors for arterial and venous thrombotic events and cerebral infarction. Autoantibodies against oxLDL were associated to coronary artery disease, myocardial infarction, peripheral vascular disease, hypertension, pre-eclampsia and diabetes. We have investigated these humoral factors in patients with rheumatoid arthritis (RA) and in individuals who developed stroke using both prospective and retrospective study designs.
We showed that the level and prevalence of IgG, IgM and IgA aCL antibodies were increased in RA patients. RA patients with extraarticular manifestations have increased levels of IgM and IgA compared to patients without these manifestations. In patients who developed stroke level of IgM aCL was slightly increased compared to referents, but aCL could not be considered as an independent predictive marker for stroke. The level and prevalence of all three isotypes of anti-oxLDL antibodies were increased in RA patients, particularly in those who had myocardial infarction, while there was no association to the development of stroke.

We have investigated the polymorphisms of genes of pro-inflammatory cytokines, such as IL-1β and TNFα and anti-inflammatory IL-1Ra that suppresses the effect of IL-1β.
We demonstrated an association of low-secretory allele A1 of TNFα to RA. The severity of RA was influenced by carriage of high-secretory allele A2 of TNFα.
Allele A1 of TNFα was associated to development of stroke in hypertensive male patients, indicating that males bearing this allele have a higher risk to develop stroke.
The study of polymorphisms of IL-1β revealed an association of genotype A2A2 to severity of RA and no association to development of stroke.
The investigation of IL-1Ra gene polymorphisms revealed that genotype A1A2 was decreased in RA patients compared to referents, while A2A3 was increased. In contrast, there was no association of IL-1Ra polymorphisms to the development of stroke.
IL-1β and IL-1Ra alleles of different haplotypes were compared between RA patients with and without cardiovascular complications. We showed that haplotypes A1+ IL-1β/A2+ IL-1Ra and A2+ IL-1β/A2+ IL-1Ra were decreased in RA patients with cardiovascular complications, indicating that A2 of IL-1Ra has a protective role by blocking the action of IL-1β.
Similarly, haplotype A2+ IL-1β/A2+ IL-1Ra was more prevalent in normotensive patients who developed stroke.
Key words: atherosclerosis, rheumatoid arthritis, stroke, autoantibodies, pro-inflammatory cytokines

CONTENTS

LIST OF PUBLICATIONS

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

I **Cvetkovic Trifunovic, J**., Wållberg-Jonsson, S., Stegmayr, B., Rantapää-Dahlqvist, S. Lefvert, A.K.
Susceptibility for and clinical manifestations of rheumatoid arthritis are associated with polymorphisms of the TNFα, IL-1β and IL-1Ra genes.
Journal of Rheumatology 2002:29:212-219

II **Cvetkovic Trifunovic, J**., Wållberg-Jonsson, S., Ahmed, A., Rantapää-Dahlqvist, S. Lefvert, A.K.
Increased levels of autoantibodies against oxLDL, MDA-LDL and cardiolipin in patients with rheumatoid arthritis.
Rheumatology, in press 2002

III Wållberg-Jonsson, S., **Cvetkovic Trifunovic, J**., Sundqvist, K.G., Rantapää-Dahlqvist, S. Lefvert, A.K.
Activation of the immune system and inflammatory activity in relation to markers of atherothrombotic disease and atherosclerosis in rheumatoid arthritis.
Journal of Rheumatology, in press 2002

IV **Cvetkovic Trifunovic, J**., Wiklund, P.G., Ahmed, E., Weinehall, L., Hallmans, G., Lefvert, A.K.
Polymorphisms of IL-1β, IL-1Ra and TNFα genes and risk factor for stroke.
Manuscript

V Ahmed, E., **Trifunovic, J**., Stegmayr, B., Hallmans, G., Lefvert, A.K.
Antibodies against oxidatively modified LDL do not constitute a risk factor for stroke. A nested case-control study.
Stroke, 1999;30;2541-2546

VI Ahmed, E., Stegmayr, B., **Trifunovic, J**., Weinehall, L., Hallmans, G., Lefvert, A.K.
Anticardiolipin antibodies are not an independent risk factor for stroke. An incident case-referent study nested within the MONICA and Västerbotten Cohort Study.
Stroke, 2000;31:1289-1293

LIST OF ABBREVIATIONS

aCL	Anticardiolipin antibodies
aPL	Antiphospholipid antibodies
apoB	Apolipoprotein B
β_2-GPI	Beta2-Glycoprotein I
CAD	Cardiovascular disease
CIC	Circulating immune complex
CRP	C reactive protein
Cu^{2+}	Copper ions
EC	Endothelial cells
ESR	Erythrocyte Sedimentation Rate
GM-CSF	Granulocyte Macrophage-Colony Stimulating Factor
HDL	High-Density Lipoprotein
HLA	Human Leukocyte Antigen
HSP60	Heat Shock Protein 60
ICAM-1	Intracellular Adhesion Molecule-1
IL-1β	Interleukin-1 beta
IL-1Ra	Interleukin -1 Receptor antagonist
INFγ	Interferon gamma
LDL	Low-Density Lipoprotein
MCP-1	Monocyte Chemotactic Protein-1
MDA	Malondialdehyde bis
MHC	Major Histocompatibility Complex
MI	Myocardial Infarction
MMP-1	Matrixmetalloproteinases-1
MONICA	Multinational Monitoring of trends and Determinants in Cardiovascular Disease
NO	Nitric Oxide
PAI-1	Plasminogen Activator Inhibitor-1
PBMC	Peripheral Blood Mononuclear Cell
PCR	Polymerase Chain Reaction
PDGF	Platelet-Derived Growth Factor
PEG	Polyethylene Glycol
PLA_2	Phospholipase 2
RA	Rheumatoid Arthritis
RFLP	Restriction Fragment Length Polymorphism
SLE	Systemic Lupus Erythematosus
SMCs	Smooth Muscle Cells
SNP	Single Nucleotide Polymorphism
TGFβ	Transforming Growth Factor beta
T_H	T helper cell
TMMP-1	Tissue Inhibitor of Metalloproteinases-1
TNFα	Tumor Necrosis Factor Alfa
TPA	Tissue-type Plasminogen Activator

VCAM-1	Vascular Adhesion Molecule-1
VLDL	Very Low-density lipoprotein
VNTR	Variable Number Tandem Repeats

1. INTRODUCTION

According to World Health Organization (WHO), cardiovascular diseases (CVD) is the name for a group of disorders of the heart and blood vessels. The following disorders are included in this group:

- Hypertension
- Coronary heart disease
- Cerebrovascular disease
- Peripheral vascular disease
- Heart failure
- Rheumatic heart disease
- Congenital heart disease
- Cardiomyopathies

The WHO report from 1999 showed that CVD are already the major cause of mortality and morbidity in Westernized countries. It was estimated that CVD are going to be the leading cause of death in developing countries by the year 2010. There are several reasons for these discouraging prognoses, but most important is change of lifestyle as a consequence of industrialization, urbanization and globalization. Some of the most important risk factors for the development of CVD are use of tobacco, unhealthy diet and physical inactivity. In poor, developing countries low birth weight, folate deficiency and infections should be included in the list of risk factors. However, this list of risk factors is growing, according to the results collected all over the world during the last decades. Discoveries of infections as a cause of atherosclerosis (both bacterial and viral agents), the probable and pathological involvement of autoantibodies against phospholipids and modified Low Density Lipoproteins (LDL) has improved our knowledge about atherosclerosis. The goals of the WHO global strategy and some major key areas in the fight against CVD are:

- planning and providing of successful national programs, which would have for goal to reduce above mentioned risk factors
- monitoring of prevention and control initiatives
- improvement of standards of care, as well as
- lowering care costs in both developing and developed countries

1.1. PATHOGENESIS OF ATHEROSCLEROSIS

Atherosclerosis is a multifactorial disorder. It entails an unregulated accumulation of lipids in the vessel wall (1). Atherosclerosis has been categorized as an inflammatory disorder, with similar chronic inflammatory conditions found in rheumatoid arthritis (RA) (1). The most important risk factors for atherosclerosis have been identified by epidemiological studies and are similar to those previously mentioned for the development of CVDs. They are presented in the Table 1.

Table 1. Risk factors associated to atherosclerosis.

Classical risk factors
Elevated levels of
- LDL & VLDL
- Lipoprotein (a)
- Homocysteine
- Fibrinogen
Low levels of HDL
Family history of premature atherosclerosis and familial hypercholesterolemia
Hypertension
Diabetes mellitus
Obesity
Male gender
Lack of physical exercise
Smoking
Infectious agents
Age

Cholesterol is an essential foundation molecule of cellular membranes, important component of blood lipids and serves as a precursor of steroid hormones. High levels of cholesterol are associated to the development of atherosclerosis. Plasma lipoproteins are macromolecular complexes of specific proteins called apolipoproteins. Apolipoproteins combine with the lipids and form spherical lipoprotein particles, which have hydrophobic lipids in the core and hydrophilic side chains of protein amino acids at the surface. Different combinations of lipids and proteins make particles of different size, density and function. There are four major classes of lipoprotein particles: chylomicrons, very low-density lipoproteins (VLDL), low-density lipoproteins (LDL) and high-density lipoproteins (HDL).

Fatty acids from diet are converted into triacylglycerols and packed together with apoB-100, apoC-I, apoC-II, apoC-III and apo-E into the VLDL particles and further metabolized into the LDL particles (2). The majority of serum cholesterol is transported in this form. These particles are very rich in cholesterol, cholesteryl esters and apoB-100. Apolipoproteins act mostly as signals, targeting lipoproteins to specific tissues and activate certain enzymes. The LDL particles are taken up into the cells via LDL receptors, thanks to the recognition of N-terminal on the apoB-100 (3).

Plasma LDL particles become susceptible to enzymatic and nonenzymatic modifications in the arterial wall when retained by extracellular matrix proteins (4). The oxidative modification of LDL *in vivo* is believed to occur in the intima of the artery wall, where macrophages and endothelial cells generate free oxygen radicals and nitric oxide (NO) in response to activating stimuli (5). Recent studies of the role of NO in the atherosclerosis gave some controversial results. The NO produced by endothelial NO synthase (eNOS) has protective, vasodilator function (6). The NO produced in macrophages, by inducible NO synthase (iNOS), is mostly responsible for potent oxidation of LDL *in vivo*. It was shown that inhibition of iNOS decreased atherosclerosis in rabbits (7). Many lines of evidence suggest that oxidative modification of LDL particles (in the lipid and apoB component) induce the initial formation of fatty streaks (8, 9).
The oxidizability of LDL depends on its size. The small particles are more susceptible to oxidation than larger (10). The extent of oxidation can range from "minimal" (mmLDL), which still could be recognized by LDL receptors (8), and extensive oxidation. Minimally modified LDL induce the expression of monocyte chemotactic protein –1 (MCP-1) and P-selectin from the human aortic endothelial cells (HAECs)(11, 12). The extensively oxidized particles are cytotoxic (13). Oxidized particles bind to scavenger receptors, that are expressed on macrophages and SMC (9). Receptor ligation activates macrophages and induces cytokine secretion (14). One of the major effects of oxidized LDL (oxLDL) is an impairment of the endothelial production of nitric oxide.

The properties of oxidized forms of LDL have been extensively studied on oxidized LDL prepared by oxidation with copper ions (oxLDL) and malondialdehyde bis (MDA) molecules (MDA-LDL). Oxidation of LDL by Cu^{2+} ions reveal neoepitopes, such as oxidized phospholipids, oxidized sterols and oxidized cholesteryl linoleat (13, 15). Reactive aldehydes generated during lipid peroxidation form covalent adducts with lysine and histidin residues of apoB and other associated proteins (16, 17). The modification of LDL by MDA involves lysines and they are considered to be one of the major epitopes for autoantibodies against MDA-LDL.
The oxidation of LDL also occurs in the circulation, but to which degree is still unknown, since anti-oxidative defense mechanisms are very active. However, oxLDL can be detected in the circulation (18, 19). According to the study of Ylä-Herttuala *et al.* (20) most of the oxidation occurs in the artery. Incubation of the LDL with various cells, like monocytes, endothelial cells (EC), smooth muscle cells (SMC) or T lymphocytes also induce oxidation (21). Beside the oxidation of LDL, there is also nonenzymatic glycation of apolipoproteins (22).

After the accumulation of extracellular lipids and separation of antioxidants from lipoproteins in the intima, accumulation of macrophages and T cells leads to the formation of the fatty streak. Recruitment of monocytes to the arterial wall and their differentiation into macrophages has mostly protective purposes. They are supposed to remove the cytotoxic and pro-inflammatory oxLDL particles and apoptotic cells. Macrophages take up accumulated lipids via scavenger receptors (23, 24) and become lipid-loaded foam cells (25) that induce the secretion of TNFα and IL-1. This recruitment is regulated by cell adhesion molecules, expressed on the arterial ECs. Adhesion molecules of particular interest are vascular cell adhesion molecule 1 (VCAM-1), intercellular adhesion molecule 1 (ICAM-1) and P-selectin.

VCAM-1 can be induced by the presence of lysophosphatidylcholine (which is a part of oxLDL) or by the hemodynamical forces (like disturbed laminar flow). When monocytes become attached via VCAM-1 to the surface of arterial EC, they penetrate the endothelial layer into the intima. As a response to the LDL oxidation, which is itself a chemoattractant, (16) different mediators are secreted from vascular wall cells, such as inducible MCP-1 (8) and pro-inflammatory cytokines TNFα and IL-1ß. The expression of both VCAM-1 and ICAM-1 is induced by these cytokines, while ICAM-1 is also induced by the oxLDL (26). The secretion of TNFα and IL-1 leads to the proliferation of SMCs, their production of collagen, formation of extracellular matrix, secretion of

platelet-derived growth factor (PDGF) and fibroblast growth factor. The last mentioned influence the formation and stability of the atheroclerotic plaque (22).

The formation of lesions from foam streaks to the more progressive plaque is a complex process characterized by the migration of SMCs (induced by the presence of PDGF) from the media layer into the intima or subendothelial space. The SMCs accumulate within the expanding intima and start producing extracellular matrix proteins, forming a fibroatheroma (1, 9). The transforming growth factor ß (TGFß) influences the production of collagen from SMCs (22). This phase of lesion formation is also influenced by the simultaneous actions of macrophages and T cells. The activated T cells produce both Th1 and Th2 cytokines (27). Surprisingly, Dansky *et al.* (28) showed that immune responses that modulate progression after formation of early lesions and secreted cytokines have both atherogenic and anti-atherogenic effects. For example, the INFγ is a Th1 cytokine that reduces the expression of scavenger receptors, inhibits SMCs proliferation and collagen production, but also stimulates production of pro-inflammatory cytokines from macrophages (3). Higher concentration of pro-inflammatory cytokines could further increase accumulation of macrophages at the inflammatory site.

The plaque rupture (especially of those with thin fibroatheroma and large necrotic cores) allow free circulation of the oxidized lipids, tissue factors and may lead to thrombosis and myocardial infarction and stroke (29, 30). Macrophages and proliferative SMC, which fail in the digestion of lipids at the site of accumulation, become apoptotic cells (31) and build up a necrotic core. Matrix proteinases, once free from necrotic cells, start with digestion of extracellular matrix proteins (32). Huang *et al.* (33) recently showed that oxLDL provokes a reciprocal regulation of metalloproteinase-1 (MMP-1) and tissue inhibitor of metalloproteinase-1 (TMMP-1). Accordingly, oxLDL stimulates endothelial collagenase activity and facilitates the neovascularization of the atherosclerotic plaques. It was shown previously that penetration of neovessels in the atherosclerotic plaque leads to better blood supply of proliferative SMCs (34) and together with protease activity, is associated with plaque rupture (35).

1.2. HYPOTHESES ON ATHEROSCLEROSIS

1.2.1. The response-to-injury hypothesis

The oldest hypothesis dates from 1856 and was presented by the German pathologist, Virchow IV, R (36). His concept was that injury induces atherogenesis. Hemodynamical factors such as high/low shear stress, reduced flow velocity, turbulent flow and increased residence time play an important role, because certain segments of arterial tree, like e.g. branching points, are more predisposed to atherosclerosis. Because of the reduced flow velocity and increased residence time, important atherogenic agents, like lipoproteins, can induce specific cell reactions from EC. The EC, when stimulated, also produce chemoattractants, growth mediators, vasocontrictors (like endothelin, angiotensin II, thromboxane A_2, arachidonic acid) and vasoactive substances (like NO, vasodilators, prostacyclin (PGI_2)). The secretion of these matrix components leads to the modification of attached lipoproteins and influences the SMC activity. The presence of all named mediators induces also the expression of surface adhesion molecules (ICAM-1 and VCAM-1), leading to attraction of T-lymphocytes, macrophages and platelets. Accumulation of these cells, as well as the accumulation of lipids inside and around SMCs, are the starts of the formation of early lesion.

1.2.2. The organized-thrombus hypothesis

This hypothesis is also quite old, dating from the nineteenth century, and was postulated for the first time by Rokitansky. He proposed that thrombogenic factors might play a role in the development of atherosclerosis. This postulation waited to be confirmed until 1957, when More *et al*. (37) described a role of mural fibrin thrombi of aorta in the pathogenesis of atherosclerosis in humans. Later, similar results were found in primates by Faggiotto, A. and Ross, R. (38). It has been speculated that platelet or fibrin deposits might be the initial event in atherosclerosis. A report from Fuster *et al.* (39) showed that activated platelets induce vasoconstriction by releasing thromboxane A2 and serotonin. Thrombosis and vasoconstriction are associated with the acute onset of myocardial ischemia, because vasoconstriction causes the plaque to rupture.

1.2.3. Infections as a cause

The presence of antibodies against viruses and bacteria in the serum or in the plaque opened the door for a completely new area in the investigation of atherosclerosis and its pathogenesis. An increasing body of evidence indicate that pathogens, especially *Chlamydia pneumoniae* are involved in the pathogenesis of atherosclerosis and increase the risk of atherosclerotic vascular diseases (40-43). Kalayouglu and Byrne found that exposure of macrophages to *C. pneumoniae* followed by exposure to LDL, caused a marked increase in the number of foam cells and accumulation of cholesteryl esters (44). IgG and IgA antibodies to *C. pneumoniae* appear in patients with carotid atherosclerosis (45) and myocardial infarction (MI) (46-48). Antibodies against this pathogens, in the form of circulating immune complexes (CIC), have been found and associated also to the development of cerebrovascular diseases, such as ischemic cerebral infarctions and transient ischemic attacks (49, 50).

Several studies from Wick's laboratory (51-54) have led to proposal of the hypothesis that the host immune response to microbial Hsp60 may lead to the development of atherosclerosis. Burian *et al*. showed that high levels of antibodies against human Hsp60 and *C. pneumoniae* are independent risk factors for coronary events and their simultaneous presence increases the risk for disease development (43). Huittinen *et al.* (55) have recently showed that autoimmunity to human Hsp60 was a risk factor for coronary events. Elevated levels of autoantibodies against *C. pneumoniae* were good predictors of coronary events several years before the event, especially if present together with *C. pneumoniae* infection (55).

1.2.4. Autoimmunity

The idea that an infectious agent could trigger the development of autoantibodies against self-antigens opened a door for a new hypothesis. A prerequisite for vascular autoimmunity is a humoral or cellular immune reactivity to self-antigens. Modified and cross-reactive antigens in the lesions, like oxidized forms of LDL (56) and/or cardiolipin suggested out that an autoimmune attack is taking place in the vicinity of atherosclerotic plaques. Lesions themselves are of an inflammatory nature. The presence of inflammatory cells (57-59), pro-inflammatory cytokines (60, 61) and the link between acute phase reactants and atherosclerosis give enough evidence to conclude that the inflammatory response in atherosclerosis is intensive. High levels of C reactive protein (CRP), which is a marker of inflammation, has been already associated to atherosclerosis (62). β_2-Glycoprotein I (also known as apolipoprotein H) is a plasma protein that binds to phospholipids. It is considered as a "co-factor"(63) and it is most likely a target of antiphospholipid (aPL) antibodies. Some studies suggest that it is required for reaction of aCL antibodies and for cardiolipin/phospholipid interaction. It was also postulated that binding of β_2-GPI to phospholipids reveals some cryptic epitopes to which aPL antibodies react. High levels of antibodies against β_2-GPI have been associated to atherosclerosis (64, 65). Protein C and S normally promote fibrinolysis and inactivate the clotting system. β_2-GPI can inhibit protein C activation *in vitro* (66) and it is believed that this co-factor may inhibit phospholipid-dependent activation of protein C by occupying the phospholipid surface. Antibodies against other co-factors, protein S or prothrombin (67) have also been associated to atherosclerosis. Their pathogenicity is still not clear. Correlation of different autoantibodies to development of atherosclerosis, as well as the presence and importance of other autoimmune factors will be discussed in more detail.

1.3. AUTOANTIBODIES AGAINST CARDIOLIPIN AND OXIDIZED FORMS OF LDL

Cardiolipin is a phospholipid originally discovered in 1941 (68) when the Wasserman serological test for syphilis revealed the presence of antibodies reactive to antigen from bovine heart tissue. After the discovery of these antibodies, cardiolipin has been isolated and studied. It is a dimeric phospholipid (diphosphatidylglycerol), where two diester phosphates are linked by glycerol (69). This compound is localized to the mitochondrial wall and as such is important in the regulation of the proton gradient across the inner membrane (70).

Cardiolipin appeared quite early in the evolution and is present in many different eukaryotic organisms. The concentration and the role of the cardiolipin could be different from that found in mitochondria (70). With the discovery of antibodies against cardiolipin, a wide range of other antibodies, with remarkable effects on the coagulation, were discovered.

Antiphospholipid antibodies are a group of antibodies that recognize epitopes on protein-phospholipid complexes. Some of them bind to anionic phospholipids, like cardiolipin or to cofactors like ß$_2$-GPI. Others bind

to neoepitopes, which are revealed when complex protein-phospholipid is formed, after the oxidation of phospholipids alone or in the combination with proteins. This group of antibodies includes antibodies against phosphatidylserine and phosphatidylethanoamine. These have been already linked with inflammatory and autoimmune disease like Systemic Lupus Erythematosus (SLE) (71). Papers in this book have investigated the presence of the aCL and antibodies against oxidatively modified LDL. Therefore, most attention will be given to these two groups.

Antiphospholipid antibodies are associated with hypercoagulability. The presence of aCL was associated with thrombocytopenia (72), recurrent thrombosis (73), repeated fetal loss and lately to CVD (74, 75). Anticardiolipin antibodies have been considered as a risk factors for arterial and venous thrombotic event (76) and cerebral infarction (77). The prospective Helsinki Heart Study revealed that aCL could be classified as a independent risk factor for myocardial infarction or cardiac death in dyslipoproteinemic middle aged men (78). Wu *et al.* (79) showed that in a prospective study where subjects of study were healthy men 50 years of age.
Patients with SLE have a nine-fold higher mortality than expected from population-based rates (80). The occurrence of aCL has been linked to myocardial infarction in young patients with SLE (81, 82). Svenungsson *et al.* (83) recently showed that both aCL and antibodies against ß$_2$-GPI are associated with arterial disease in SLE patients.

Several mechanisms have been proposed for the pathogenesis of aPL antibodies. They have been correlated to inhibition of activated factor V (84), platelet activation (85, 86) and aggregation (87). Anticardiolipin antibodies were found in the subendothelial cardiac deposits (88). They promote interaction between endothelial cells and platelets. Studies in vitro showed that the β2-GPI-aCL complex activates endothelium (89), and induces up-regulation of adhesion molecules, like ICAM-1 (90). The endothelial damage exposes anionic phospholipids that in combination with β2-GPI or prothrombin are targets for autoantibodies. It is interesting that LDL itself may be a target of aCL (91) or in the combination with β2-GPI (92). A major antigen of aPL is the co-factor β_2-GPI. It was suggested by Matsuura *et al.* (93) that β_2-GPI can also bind to oxLDL, oxVLDL or oxHDL that are produced by Cu^{2+} oxidation.

As previously described, oxidation of LDL is one of the most important events in atherogenesis. The modifications of the lipid and proteins of LDL reveal antigenic epitopes, which are inducers of different autoantibodies. Autoantibodies to epitopes of oxLDL were found in both human serum (15) and in the atherosclerotic plaques (94). In addition, these autoantibodies were also present in lesions, partly in the form of immune complexes with oxLDL. Since than, several studies showed a correlation between autoantibodies against oxLDL and cardiolipin. These autoantibodies were found both in patients with cardiovascular diseases as well as in apparently healthy populations. Some of the associated diseases are coronary artery disease, myocardial infarction, peripheral vascular disease, hypertension, pre-eclampsia and diabetes. Salonen *et al*. 1992 (95), used the MDA-LDL as a model epitope of oxLDL. They found that autoantibodies against MDA-LDL could be good predictors of progression of carotid intima-medial thickness in healthy males. Recently, Vaarala *et.al* (65) suggested that autoantibodies to modified LDLs and other phospholipid-protein complexes could be used as markers of cardiovascular diseases.

Elevated levels of autoantibodies to oxLDL have been demonstrated in patients with atherosclerosis (79, 95, 96) and could predict the accelerated atherosclerosis. The pathogenesis of these antibodies were investigated by *in vitro* study on macrophages and it was showed that they could increase accumulation of LDL into macrophages by a Fc-receptor mediated mechanism (97). These antibodies show some cross-reactivity with autoantibodies against cardiolipin due to their binding to common epitopes on the oxidized lipids (98, 99). Antibodies against oxLDL and MDA-LDL have been described in patients with SLE (99, 100) and in the aPL syndrome (101). Antibodies to oxLDL bind to lipid-protein complexes and are cross-reactive with aCL. There is also subpopulations of antibodies to oxLDL that do not cross-react. It is suggested that these antibodies probably recognize epitopes on the apoB of

modified LDL (102). According to studies of Aho *et al.* (103) and Amengual *et al.* (101), antibodies to oxLDL do not interfere with blood coagulation, as aCL do. Therefore, these antibodies could be considered as markers of autoimmune arterial disease.

1.4. PRO-INFLAMMATORY CYTOKINES IL-1β, TNFα AND ANTI-INFLAMMATORY IL-1Ra AND THEIR GENE POLYMORPHISMS

Cytokines are low molecular weight proteins (ca ~8-80 kDa) that represent a functional bridge between innate immunity and antigen-specific adaptive immunity. Cytokines can be produced by various cell types. They act by binding to specific receptors on the membrane of target cells. Their binding to the receptors enhances/inhibits the expression of others genes and often influence each other. Several different cytokines can function similarly or their action can be simultaneous.

The $CD4^{+}T_H$ cells have two different profiles according to cytokine production. Typical T_H1 cytokines are Interferon-gama (IFNγ) and Interleukin-2 (IL-2). These cytokines activate macrophages to secrete pro-inflammatory cytokines IL-1 and Tumor Necrosis Factor β (TNFβ). Already activated macrophages secrete IL-12 (104, 105) and that induces the T_H1 cytokine pattern.

Studies of Geng *et al.* (106), Hansson *et al.* (57) and Uyemura *et al.* (107) showed that atherosclerotic plaques contain INFγ (106) and IL-12 (107, 108). It was also found that T cells from plaques recognize oxLDL (108) and that LDL oxidation induces activation of macrophages characterized by the secretion of IL-12 (107). Frostegård *et al.* (60) found that IL-1 and GM-CSF in atherosclerotic plaques are secreted from macrophages, endothelial cells and some SMCs and confirmed that the T_H1 pathway is dominant in atherosclerosis.

The function of TNFα is similar to the function of IL-1, but TNFα also induces secretion of IL-1 (109). OxLDL induces secretion of TNFα in SMC (110) and this cytokine induces the proliferation of SMC in plaques (111). Together with IL-1, it stimulates expression of adhesion molecules in EC (59). It seems that the progression of atherosclerotic lesions depends on the balance between pro-inflammatory and anti-inflammatory cytokines (Th1/Th2 cell balance).
The IL-1 family of cytokines includes two agonists, IL-1α and IL-1β and a specific antagonist, IL-1Ra. While IL-1α and IL-1β have a pro-inflammatory role in the pathophysiology of various human diseases, IL-1Ra is an anti-inflammatory cytokine that exists in three isoforms. Two isoforms are intracellular (icIL-1Ra), while third is a secretory (sIL-1Ra) (112), (113), (114), (115). The sIL-1Ra is a secreted product of monocytes, macrophages, neutrophils and other cells, while intracellular forms are constitutinally secreted in keratinocytes and epithelial cells (113, 116). IL-1Ra is a structural variant of IL-1 and binds to both types of IL-1R, but can not promote the signaling pathway as IL-1 (112, 117). When IL-1 binds to the surface receptor IL-1RI, a long cytoplasmic tail from this receptor together with accessory protein (IL-1AcP) initiates cell stimulation. However, IL-1Ra prevents binding of IL-1AcP, thus producing no response.

The levels of IL-1 and TNFα are high in the rheumatoid synovium. These cytokines have both local and systemic effects in RA. Locally, they induce the expression of adhesion molecules on endothelial cells, enhance the migration of leukocytes to the inflamed sites and, most importantly, induce the degradation of joint tissue. The degradative processes are induced by actions of released enzymes (metalloproteinases) from synovial fibroblasts and chondrocytes. Locally, they induce production of oxygen radicals and reactive nitrogen intermediates and inhibits the production of matrix components.

Systemic effects of these cytokines are achieved by the induction of synthesis of acute phase proteins from hepatocytes. The IL-1Ra cytokine is considered as an acute-phase protein derived directly from the liver. High levels of IL-1Ra are present in the circulation of patients with acute (118) and chronic inflammatory diseases (119) and after surgery (120).

The importance of maintaining an adequate ratio of IL-1Ra to IL-1 in both prevention or treatment of different diseases (especially RA) has been established in studies on different transgenic and knock-out mice described later. Studies of the relationship between polymorphisms of these cytokine genes and the severity of some rheumatic diseases have also been a hot spot of investigations (121). The study of Blakemore *et al.* (122) and Tjernström *et al.* (123) showed that SLE patients have increased frequency of allele A2 of IL-1Ra and that this allele influenced the severity of the disease. Santtila *et al.* showed that allele A2 of IL-1Ra is associated with enhanced production of IL-1β *in vitro* from mononuclear cells (124). Buchs *et al.* (125) showed that polymorphism +3954 may be an important marker for the severity of RA and associated with an imbalance between IL-1β and IL-1Ra.

The results of two recent studies of IL-1Ra knockout mice showed that maintenance of a balance between IL-1 and its antagonist IL-1Ra may be important for prevention of the development of inflammatory diseases (126), (127). When IL-1Ra knock-out mice were bred on the BALB/cA background they spontaneously developed inflammatory arthritis with many features similar to RA in humans (126). Surprisingly, the same mice bred on MFI x 129 background developed polyarteritis nodosa, an arterial inflammatory disease (127).

A disrupted balance between the levels of pro-inflammatory and anti-inflammatory cytokines and their association with several inflammatory and autoimmune diseases started a new field of investigation – genetic polymorphisms of the cytokine genes.
Genes of the IL-1 cytokine family are located as a cluster on the chromosome 2q. This gene cluster contains many polymorphic sites. Some of them are more interesting because they have been associated with higher secretion of these important cytokines. Because of its anti-inflammatory effect that could be used for therapeutic purposes, IL-1Ra gene polymorphisms are one of the most studied in this gene family. The IL-1Ra gene is located on 2q12-13, close to the IL-1α and β gene forms (128). Tarlow *et al.* 1993 (129) described an 86-bp repeat sequence in the intron 2 that occur four, two, five, three and six times and in that way determines alleles 1-5. This sequence has three potential protein-binding sites, which could be of biological significance. Allele 2 of the variable nucleotide tandem repeats (VNTR) polymorphism is associated with many inflammatory and chronic diseases (alopecia areata (130), SLE (122) and ulcerative colitis (131)). This allele might be associated with locally higher production of the cytokine, although results are inconsistent. The study of Clay *et al*. (132) could not find evidence that IL-1Ra mRNA level is dependent on the presence of allele 2. However, Danis *et al.* (133) described a relationship between allele 2 and increased production of both IL-1Ra and IL-1α in serum, while Santtila *et al.* (124) found an association with enhanced secretion of IL-1β *in vitro*.
Similarly, also the IL-1β gene became very interesting to investigate. There are several polymorphic sites in this gene and a bi-allelic polymorphism in the exon 5, a TaqI Restriction Fragment Length Polymorphism (RFLP) is of special interest. The allele 2 is associated to higher production of IL-1β (134, 135).

Recently, Tseng *et al.* (136) reported a novel polymorphism with three nucleotide substitutions in the IL-1Ra VNTR 2-repeat allele. One of the substitutions corresponds with the fourth 3' end nucleotide of the reverse primer that has been often used for the study of polymorphism in this locus. They suggest that mismatching between primer and the 2-repeat allele can cause misleading amplification results when stringent conditions are used for annealing. These new findings might be of importance.

The TNFα gene is located on the chromosome 6 between HLA-B and HLA-DR genes (137). An adenine (allele 2) instead of guanine (allele 1) at position -308 is associated with high production of TNFα by peripheral blood mononuclear cells (PBMC) of healthy people (138).

1.5. RHEUMATOID ARTHRITIS

Rheumatoid arthritis (RA) is a systemic disease characterized by inflammation of synovial tissue of joints. The chronic inflammation leads to the degeneration of cartilage and bone and surrounding tissue. RA has an incidence of ~0.1-0.2/1000 in males and 0.2-0.4/1000 in females (139). Several studies performed in Western countries of Europe, in USA or Australia showed a prevalence of RA of 0.5-2% in individuals older than 15 years. Females are more prone (2-4 times) to RA than males (140). There are some populations, such as a certain population of Indians from North America (Pima Indians) in whom the prevalence reached 5% (140).

In 1988 the American Rheumatism Association formulated the new revised criteria for the classification of RA (141). RA is defined by the presence of four or more criteria and criteria 1 through four must be present for at least 6 weeks.

- morning stiffness in and around joints lasting at least 1 hour before maximal improvement;
- soft tissue swelling of 3 or more joint areas
- swelling of the proximal interphalangeal, metacarpophalangeal, or wrist joints
- symmetric swelling
- rheumatoid nodules
- the presence of rheumatoid factor
- radiographic erosions and/or periarticular osteopenia in hand and/or wrist joints

Infectious agents and lifestyle factors have been suggested as possible causes important for the development of RA. The hypothesis, that the combination of infectious agents with a certain genetic background could be the triggering step in the development of the disease, is still valid although it will be necessary to explain the pathological mechanism. Very interesting findings of Albani *et.al* (142) and Auger *et.al* (143) showed that the *Escherichia coli* chaperone proteins induce a cellular immune responses. The response was higher in synovial fluid than in the blood and higher in children with active juvenile RA than in those in remission. These studies show that increased immune responses against common bacteria may contribute to the generation of chronic inflammation in juvenile RA and that the magnitude of the immune response is linked to the disease activity.

Several studies have suggested that RA is partly of genetic origin and that development of disease or its severity could be influenced by the presence of certain genetic markers. The evidence, that certain genetic background could be a cause for RA, was first found in twin studies. Aho *et.al* (144) showed that disease concordance for RA in Finish population was 12% for monozygotic twins compared to 4% in dizygotic twins. Seven years later, Silman *et.al* (145) showed that disease concordance for RA in monozygotic twin pairs from UK reached 15%. Subsequent studies showed that susceptibility to RA and its severity is associated with the class II genes in the major histocompatibility complex loci encoding for HLA-DR molecules. HLA DR1 and HLA DR4 have been associated to rheumatoid arthritis, DR2 and DR3 are associated to SLE, while HLA class I B27 is associated with ankylosing spondilitis (146).

The TNFα gene polymorphisms and the action of this cytokine have been intensively investigated because of its pivotal role in the pathogenesis of RA. Because of the gene position on chromosome 6, between HLA B locus and MHC class II DR genes, this cytokine is well studied in patients with different connective tissue diseases. The major sources of the TNFα are macrophages and T cells and the first evidence on interindividual variations in the production of this cytokine came from the study on SLE patients (147). The presence of HLA-DR2 was associated with lower production of TNFα following the stimulation with LPS. Studies on cell lines showed that haplotypes crossing the MHC class I and II, such as HLA A3-B7-DR2-DQ1 and HLA A1-B8-DR3-DQ2 are probably inherited as a group (148). It is likely that any variations in the class III region will involve also the TNF locus. Studies of microsatelites in the TNF region confirmed that TNFb3-TNFa2 and TNF-308 allele A2 (also called A-allele) are linked (149, 150).

Association with the MHC DRβ1 alleles that share the amino-acid sequence at position 70-74 in the binding groove of the MHC has been investigated in RA patients of Caucasian origin (151). Gregersen *et.al* (152) showed that HLA genes associated with RA contain a certain amino acid sequence in the third hypervariable region of the DR ß chain. This shared epitope shows some ethic and geographical variations. Although there are many studies showing the association of the polymorphisms from this locus to RA, there are also reports with conflicting results. In the populations from northern Europe alleles DRB1*0401, *0404 and *0408 are associated. In the populations from southern Europe alleles DRB1 *0405, *0101, *0102 and *0101 are more common in RA patients (153-157). The question how genetic factors determine an individual's susceptibility or the severity of the RA remains to be answered.

Patients with the most severe joint disease (as defined by the Sharp index) develop early damage with rapid progression. The GG genotype of the polymorphism -238 in the TNFα gene was investigated in patients with RA in a prospective study and was associated with faster progression, while patients with genotype GA had a slower progression (158). The presence of HLA DR4 together with the -238 GG genotype increased the odds ratio for the presence of bone erosions.

Stimulated PBMCs from HLA DR3 and HLA DR4 positive SLE patients had more production of TNFα than cells from DR2 positive cases (147). Several *in vitro* studies investigated the association between the -308 polymorphism and the production of the TNFα. In patients with chronic reactive arthritis, the common G allele (allele A1) was associated with reduced levels of TNFα in HLA-B27 positive patients (159). Recently, Rudwaleit *et al.* (160) showed that the T cell production of IFNγ and TNFα in healthy individuals positive for HLA-B27 and genotype A1A1 of the -308 polymorphism was significantly reduced. When one copy of allele A2 was present, these individuals produced higher levels of TNFα. Also, it was shown that patients with ankylosing spondylitis had similar production of the cytokine in response to HLA-B27 and A1A2 -308 positivity. Wilson *et al.* reported that cell lines with a construct that included the -308A allele produced six times more TNFα mRNA than cells with constructs including the -308G allele (161). Similar results were found by Kroeger *et al.* investigating the same polymorphism in Jurkat (T cells) and U937 (macrophage) cell lines (162).

The pathophysiological processes that lead to clinical symptoms of RA involve also the overproduction and overlapping functions of both TNFα and IL-1 (117, 163). Eastgate *et al.* found that patients with active RA have increased concentration of IL-1β in the plasma (164). Both cytokines increase the levels of cyclooxygenase 2, prostanglandin E_2, NO, adhesion molecules, IL-6, chemokines and collagenases and promote procoagulant activity (165). The TNFα increases levels of IL-1β and transmits the T_H1 response, while IL-1 is a comitogen for T lymphocytes and increase the secretion of TNFα (166). The persistent activation in chronic disease, like RA, induces metabolic abnormalities and the destruction of the tissues. The IL-1 induces the accumulation of inflammatory cells in the synovium. These cells further produce additional messengers, like proteoglycans and proteases that lead to the formation of pannus. Increased levels of IL-1β in the synovium up regulate

metalloproteinases (MMP) resulting in the erosions of the joints. Therefore, polymorphism of the IL-1β gene has been a subject of intensive research (125, 167-169). Some of these studies found an association of polymorphisms in this gene to the severity of RA (125, 168). Carriage of the rare allele A2 of the polymorphism at the position +3954 in the IL-1β gene was increased in destructive arthritis and severity of the disease was related independently from the HLA-DR4/DR1 haplotype (125). However, Huang *et al.* did not found the association of the either IL-1β or IL-1Ra gene polymorphisms in Taiwanese RA patients (169).

Polymorphisms of the IL-1Ra gene and its association to the susceptibility and severity of RA was studied in the previously mentioned investigations of IL-1β. The balance between IL-1 and IL-1Ra is important in many autoimmune diseases (117, 121). IL-1Ra have an anti-inflammatory, beneficial effect in RA patients (proved by the successful drug treatments with IL-1Ra (170)). Treatment of RA with IL-1Ra resulted in reduced mononuclear cell infiltration of synovial membrane. However, Vencovsky *et al.* showed the association of the rare IL-1Ra "high producing" allele 2 to the development of juvenile idiopathic arthritis (171).

Many clinical studies have suggested that RA is associated to increased mortality. There are several studies of mortality in RA from USA (172), Australia (173), Sweden (174) and Norway (175). Several epidemiological studies showed that CVD is more frequent in RA patients than in the population in general (175-178) and that patients die at an earlier age (173, 179, 180).
The acute and chronic disease of RA may lead to cardiac disease by mechanisms of vasculitis, nodule formation and fibrosis (181).

The incidence of thrombosis is higher in RA patients than in healthy individuals. Some authors suggest that an altered lipid profile, such as increased lipoprotein (a) and the presence of aCL represent an important risk factor (182). Fort *et al.*1987 found a significantly elevated frequency of aCL levels in patients with SLE (38%), RA (33%), and psoriatic arthritis (28%), but no correlation between aCL levels and recurrent thrombosis.

Ginsberg *et al.* 1993 proved that aCL are associated with a prothrombotic state in SLE patients. They analyzed prothrombin fragments (F1 and F2) and fibrinopeptide A (FPA), as indices of thrombin generation and activity, respectively, and aCL (183). Seriolo *et al.* 1999 observed the association of aCL and decreased levels of free Protein S with higher rates of venous and/or arterial thromboses in patients with RA compared to controls (184). A year later, the same group reported significant increases of plasma homocysteine levels in RA patients with thromboses (185). According to that study, association of aPL (aCL and lupus anticoagulant) and high levels of plasma homocysteine, represent a risk factor for thrombotic events. These might be involved in the high mortality of RA patients with a history of thrombosis. Several studies confirmed association between high levels of serum homocysteine and stroke (186).

Phospholipases A_2 (PLA_2) are a group of enzymes responsible for the mobilization of arachidonic acid and other fatty acids from membrane phospholipids (187, 188). Arachidonic acids mobilized by the action of $sPLA_2$ is the substrate for the biosynthesis of several lipid mediators of inflammation, including prostaglandins, thromboxane and leukotriens (189). These mediators are important also in the development of atherosclerosis. Recently, Triggiani *et al.* showed that soluble PLA_2 induced concentration-dependent secretion of TNFα and IL-6 from blood and synovial monocytes from RA patients (190) and that $sPLA_2$ activate pro-inflammatory cytokines.

1.6. STROKE

Ischaemic stroke could be defined as a cardiovascular disease that affects blood vessels that supply the brain with blood. The usual cause for stroke is atheroclerotic damage of blood vessel. Bursting of blood vessels, formation

of a local thrombosis and release of a blood clot, can lead to the blockade of cerebral blood flow. The necrosis of the brain tissue behind that rupture or blockage leads to the neurological deficit (22).

According to the Northern Sweden MONICA study (Multinational Monitoring of Trends and Determinants in Cardiovascular Disease) the incidence of stroke (first ever stroke) per 100, 000 in the age group 25-74 years has varied little over the years. The study was monitoring the incidence of stroke in both women and men according to strict WHO criteria. During the period from 1985-1998 in total 5 590 men and 3 541 women had a first ever stroke. The lowest rate in men was 232 and the highest 273, while these values in women were 139 and 184. The 28-day case-fatality decreased in both men and women from 19% to 11% (191). The data collected from this 14-years study indicate that there is no trends in the incidence of the first ever stroke event and that fatality is significantly decreased (191). The decrease of fatality could be explained by better treatment of stroke during the last decade.

During the past several years a number of studies have been conducted to identify the genetic factors involved in ischemic stroke. However, the results obtained are not consistent (192). Many genes and their polymorphisms have been investigated, like angiotensin-converting enzyme, angiotensinogen, apolipoprotein A, factor VII, prothrombin, plasminogen activator inhibitor-1, factor V, platelet activating factor, fibrinogen, methylene tetrahydrofolate reductase (MTHFR), lipoprotein lipase and endothelial constitutive nitric oxide synthase. However, there are no studies on polymorphisms of pro-inflammatory cytokines, such as IL-1β, IL-1RA and TNFα.

There is evidence that both acute and chronic infections contribute to the risk of ischaemic stroke, particularly in young patients (193, 194) and children (195). In young patients with stroke, cervical artery dissection (CAD) is an important cause of stroke (194). Infection and inflammation can induce a procoagulant state and can injure the arterial wall. Recently, Grau *et al*. (196) investigated whether young patients, who had suffered from ischaemic stroke and had history of CAD, showed an enhanced release of pro-inflammatory cytokines. The analyses involved PBMC stimulated with LPS. They found that patients with this history had a persistently higher secretion of chemokine IL-8 and a tendency to higher secretion of TNFα and IL-6.
Pro-inflammatory cytokines are also found within CNS (196-198). Cytokines in the CNS originate not only from the immune cells of the brain, but also from astrocytes and neurons (199). The action of cytokines in this tissue is via cytokine receptors on the glial cells and neuronal cells. As a direct consequence of the ionic imbalances and free calcium accumulation, there is a release of free fatty acids and other pro-inflammatory lipid metabolites associated with ischemic injury. These agents promote the release of pro-inflammatory cytokines, like IL-1 and TNFα that further promote secretion of IL-6 and IL-8, infiltration of leukocytes and production of anti-inflammatory cytokines IL-4 and IL-10. Experiments on rodents showed that IL-1 is an important mediator of neurodegeneration induced by experimental cerebral ischemia or excitatory or traumatic brain injury (200, 201). A recent report by Boutin *et al.* (202) showed that simultaneous deletion of both IL-1β and IL-1α genes caused a marked reduction of ischemic brain damage. The investigation also showed (by RT-PCR in the IL-1β knock-out mice) that IL-1β regulates expression of IL-1α (203, 204). In addition, injection of recombinant IL-1Ra (intracerebroventricularly) in wild type mice reduced the ischemic infarct volume by ~35%. In mice lacking both IL-1β and IL-1α, reduction was significantly greater (approximately 70 to 80%). The difference in ischemic damage may reflect poor brain penetration of IL-1Ra or instability of the same protein in the brain (202).

Carlson *et al.* (205) recently showed that inflammatory cytokines like IL-1α, IL-β, IL-6 and TNFα provide neuroprotection from the toxic influx of calcium, so called excitotoxicity, that is a major contributor to neuronal death induced by stroke. It seemed that in some cases the pro-inflammatory cytokines in the combination with other risk factors for stroke might have a both positive and negative effect on the development of the stroke or its

severity. New ways of treatment using blocking of these cytokines are under the clinical trials. Therefore, polymorphisms of these genes should be properly investigated.

A close association between cerebrovascular disease and aCL has been observed in well-controlled prospective studies (206). The concept of β2-GPI, as the specific target of a subset of aCL, has evolved from a series of independent studies since 1990 (207). Fiallo *et al* (208) have recently reported a higher prevalence of anti-β2-GPI antibodies in unselected patients with stroke. In patients with ischaemic stroke, there were different antibody populations (against aCL and β2-GPI) with partial overlapping in their specificity. This confirmed that antibodies against β2-GPI are associated with ischaemic neurological disorders.

2. AIMS OF THE STUDY

The main aim of this thesis was to elucidate the following questions:

1. Is the CVD in RA patients caused by the same factors as in patients who developed stroke?
2. Are certain "high-secretory" alleles of pro-inflammatory cytokines associated to RA or stroke?
3. Are autoantibodies against cardiolipin and modified forms of LDL associated to RA and stroke?
4. Would it be worth-while to use screening for these inflammatory factors as a tool for predicting of the disease or its development?
5. Would knowledge of these factors be of use for treatment?

3. METHODOLOGY

3.1. CLINICAL MATERIAL

Patients included in these studies belonged to two distinctive groups: group of patients with RA (I, II and III) and patients who developed stroke (IV, V and VI).
The RA patient group of paper I contained 152 individuals (42 men and 112 women) from Northern Sweden recruited at the Department of Rheumatology, University Hospital, Umeå. A detailed description of these patients is presented in the paper. The sera from 121 of these patients were used in the study presented in the paper II. The control population used in the paper I and III are partly from the large prospective MONICA study that was described earlier. In total, 44,725 men and women from MONICA and Västerbotten Intervention Program (VIP) were followed from 1 January 1985 to 31 August 1996. These two counties in the Northern Sweden are populated with 510 000 inhabitants, who have a unique lifestyle and dietary habits. There were 226 controls from this prospective study and 131 individuals randomly selected from a population register of the county (paper I). Table 2 presents female and male referents included in the analysis of polymorphisms of three cytokine genes.

Table 2. Patients and referents with RA and stroke included in paper I and III.

	IL-1ß		*IL-1RA*		*TNFα*	
Patients						
RA	Females	112	Females	112	Females	112
	Males	42	Males	42	Males	42
Stroke	Females	51	Females	31	Females	34
	Male	51	Males	57	Males	63
Referents						
MONICA study	Females	67	Females	70	Females	76
	Males	101	Males	103	Males	117
Population register	Females	29	Females	29	Females	96
	Males	10	Males	0	Males	35
Total referents	*Females*	*96*	Females	99	Females	172
	Males	111	Males	103	Males	152
		207		202		324

The RA patients in the paper III, in total 39 of them (9 men and 30 women), were all registered at same department between 1974 and1979 and included in an earlier study on predictors of CVD and overall mortality

(177). They were matched according to age and gender with individuals randomly selected from the population register of the same region.

Papers IV, V and VI include patients who develop stroke and were part of the prospective MONICA and VIP studies. In total 123 patients were involved. Two referents for each case were selected, in total 246 and matched according to sex, age and geographical region. Paper VI included all patients and 241 referents. Experimental work performed for purpose of several studies reduced the samples, so our study, presented in the paper V included 119 patients and 233 referents.
For the genetic part of the study in the paper IV we had access to DNA samples from 113 patients. There were 91 with brain infarction, 19 with intracerebral hemorrhage and 3 with unspecified stroke. Seventy-one were men and 42 women.
Analysis of the polymorphism in the IL-1 β gene was performed in 91 cases and 168 referents, IL-1Ra gene polymorphism in 88 cases and 173 referents and TNFα gene polymorphism study in 105 cases and 193 referents.

3.2. ISOLATION AND OXIDATIVE MODIFICATIONS OF LDL

For the analysis of autoantibodies against oxidized forms of LDL we used both copper (Cu^{2+}) (209) and MDA modified LDL (210). According to many previous studies, oxidation of LDL *in vivo* reveals many epitopes (211). This molecule is large, containing both phospholipids as well as proteins (212). Autoantibodies can be specific for either part or the combination of both. There are also differences in the oxidative events that occur in the atherosclerotic plaque compared to the oxidation that may occur in the circulation. The LDL found in the atherosclerotic plaque is surrounded with many different cells (SMC, macrophages and neutrophils). By using both copper and MDA oxidized forms we tried to achieve different epitopes to which autoantibodies may react and to mimic actual *in vivo* reactivity.
The MDA is a highly reactive dialdehyde generated during the catabolism of arachidonic acid that occur in platelets or as a result of lipid peroxidation that occurs during the phagocytosis by macrophages. It could also be a product of nonenzymatic lipid peroxidation of unsaturated fatty acids (213). Several reports have revealed that MDA-LDL is a major B-cell epitope of oxLDL in atherosclerosis (15, 56, 214).

3.3. ENZYME-LINKED IMMUNOSORBENT ASSAY (ELISA)

In the papers II, III, V and VI we used ELISA to determine the presence and levels of autoantibodies against oxLDL, MDA-LDL and aCL. We chose ELISA because it is a sensitive method that give the possibility to screen many samples at the same time and relatively cheap. The advantage of ELISA is that it is simple to perform, with good reproducibility and with no requirements for radioactive isotopes or expensive equipment.

In the case of aCL, we used the method by Haris *et al.* (215), but with some modifications as described in papers. We used 96-well microplates of polyvinylcloride that provided a good binding of cardiolipin.

Serum samples from patients and corresponding referents (age- and sex-matched) were analyzed in the same plate. Each plate contained a previously confirmed positive and negative serum sample, as well as the blanks. All samples were run in duplicates and mean optical density (OD) was used in calculations. For calculations of the prevalence we used Mean ± 2SD (standard deviation) and the values above 95% confidence interval of the referents as cutoff level. Use of this cutoff level does not hide skewness of our data.

3.4. ISOLATION OF CIRCULATING IMMUNE COMPLEXES

In order to obtain more accurate levels of free immune complexes (IC) in the serum (not bound to erythrocytes), it is necessary to perform the assay on the samples that have not been freezed and thawed for several times. Frequent thawing of serum may induce the formation of aggregates of ICs.
We used the precipitation of CICs by polyethylene glycol (PEG) performed in low concentrations of PEG (4%). The precipitation in this condition decreases the precipitation of monomeric immunoglobulins and allows the precipitation of complexes formed *in vivo*. A disadvantage is that lipoproteins and other macromolecules may precipitate as well. The purpose was to detect the presence and levels of CICs, not their isotypes or components, although this method can be used for further immunochemical and biological characterization.

3.5. ISOLATION OF THE DNA

For genetic studies in the paper I and IV, the DNA was extracted from cells from peripheral blood using the classical proteinase K digestion followed by phenol/chloroform extraction.

3.6. POLYMERASE CHAIN REACTIONS (PCR)

The polymerase chain reaction (PCR) is a very specific, primer-directed enzymatic amplification of specific target DNA sequences (216). In order to obtain a specific DNA fragment of interest, two oligonucleotide primers flanking the DNA segment are used. Primers used for the analysis of IL-1β, IL-1Ra and TNFα, as well as the description of the PCR conditions and enzymatic digestions by TaqI and NcoI are given in papers I and IV.
The most important advantage of the PCR is that it is highly specific method that gives a sufficient amount of the target DNA for further analysis by standard molecular biology techniques.

3.7. ANALYSIS OF HAEMOSTATIC FACTORS AND ULTRASOUND MEASUREMENTS

The aim of the study presented in the paper III was to investigate the relation between markers of atherogenesis and thrombogenesis in patients with RA, as well as to relate these variables to markers of inflammation and endothelial activation. The ultrasound measurements were used to reveal the presence of atherosclerotic plaques. Several haemostatic factors (von Willebrand factor, PAI-mass, D-dimer, IL-6, IL-2sRα, sICAM-1, sE-Selectin) were determined by ELISA. The functional activity of PAI-1 and tPA was analyzed by BIA, while homocysteine was determined by high performance liquid chromatography (HPLC). Measurements of all above-mentioned factors as well as the ultrasound measurements were performed at the Departments of Rheumatology and Clinical Immunology, University Hospital, Umeå.

4. RESULTS AND DISCUSSION

4.1. POLYMORPHISMS OF PRO-INFLAMMATORY CYTOKINES, IL-1β AND TNFα AND ANTI-INFLAMMATORY IL-1Ra IN RA AND STROKE

Cytokines are regulators of immune responses and have a central role in the processes of inflammation. The balance of pro-inflammatory and anti-inflammatory cytokines dictates not only the development of the disease in some cases, but also the severity. Our intention was to study two different diseases and to find similarities in the development of atherosclerosis as related to different forms of cytokine gene products. As it was previously described, genetic polymorphisms of IL-1β, TNFα and IL-1Ra were all associated to different autoimmune as well as to some cardiovascular diseases. A question arises: is it possible that certain alleles of these cytokine genes could predict/influence the development of stroke and RA? Another question was: is the influence of these polymorphic sites similar in stroke and cardiovascular complications in RA?
Results presented in the paper I and IV tried to answer these questions, but as usually happens in science, unexpected results occur and new questions arise.

4.2. THE TNFα NcoI POLYMORPHISM

Many polymorphic sites are found in the TNFα gene and for some polymorphisms, functional correlates exit (e.g. at the position –863) (217). Reports are controversial. Some polymorphisms are associated to susceptibility to RA (218), while others report association of more severe forms of RA to certain polymorphisms (219).
Common for both paper I and IV is an unusual finding that the allelic distribution was not in concordance with the Hardy-Weinberg law. In the population included in the paper IV, the estimated Hardy-Weinberg p-value was $p=0.0028$, with the $X^2=11.78$ and $df=2$. Therefore, in the study of RA patients (paper I) we included more referents to verify that this finding was not false or a random event. The referent group in the paper I included in total 324 individuals. The referent group from MONICA study included most men, while the RA patient group had more females. It is also well known that RA is more common in females. Therefore, we tried to balance the numbers of referents according to the gender. The previously presented Table 2 shows the referent group included in paper I.

Although the referent group in paper I included more referents, allelic distribution was not in concordance with Hardy-Weinberg law ($p<0.01$). This finding can be explained by confounding effects. The Northern Sweden has been sparsely populated, the population to a large extent inbred, and this leads to skewed allelic distribution. Also, the TNFα gene is located on the chromosome 6, between the HLA-B and HLA-DR genes (137). This DNA segment is highly conserved during the evolution. There are not so many recombination hot spots spread over it and recombination events occur at low levels, so haplotypes were conserved. In addition, inbreeding that occurred in this population, minimized the possibility of recombination in these DNA loci.

4.3. THE POLYMORPHISM OF TNFα IN RA

According to paper I, there was a strong association of the A1 allele to RA and allelic frequency was significantly increased in patients (p=0.0080; p_c=0.016, OR=1.63). Almost all 154 RA patients (except one) carried this allele and the high odds ratio of 23.41 indicate that this allele was associated to the development of the disease. Odds ratios were significantly high in both males and females (OR=16.30 in females; OR=13.90 in males) indicating that there is no difference between genders.
The severity of disease was influenced by carriage of so-called "high secretory" allele A2 of TNFα. The severity of the disease was presented as the accumulated activity score and calculated for each genotype. Patients with genotype A1A2 suffered from the most severe forms of RA (accumulated disease score was 5.0 for A1A2 and 4.7 for A1A1 genotype respectively) and were significantly younger at the disease onset (44.0 for A1A2 and 49.0 for A1A1 respectively). We can speculate that the low frequency of genotype A2A2, seen in this population, was caused by early death of patients with severe forms of RA and because of that not included in the study.

The recent report of Waldron-Lynch *et al*. (220) analyzed multiplex RA families and found that allele A2 at -308 is inherited with the specific TNF microsatellite haplotypes, a2, b3, c1, d1, e3 (H2). This haplotype was previously found to be associated with RA in individuals heterozygous for the HLA-DR 'shared epitope' (SE). Results showed also that A2 allele was significantly associated with RA susceptibility in individuals heterozygous for SE. This significance occurred only in patients carrying the HLA-DR4 and the SE. It was concluded that allele A2 of TNF at the position –308 plays an independent role in RA susceptibility in specific immunogenetically defined groups of RA patients. The report of Kerlan-Candon *et al.* (221) showed that the levels of surface expressed DR molecules differ on antigen-presenting cells and are correlated to certain alleles. In RA patients the density of DR4 and DR1 molecules was enhanced compared to controls and an increased of expression of DR molecules allowed a more efficient presentation of low-affinity peptides. In normal conditions, these peptides are not efficient in generating a T cell response, but in RA they could lead to activation of peripheral autoreactive T cells. On the other hand, Vinasco *et al.* (222) reported an increase of vascular noduli in patients heterozygous for A1A2. This indicates that TNFα polymorphism could have some influence on the development of cardiovascular complications found in RA, although we could not find any evidence for this.

4.4. THE TNFα POLYMORPHISM IN STROKE

In patients who developed stroke (paper IV), we found an association of genotype A1A1 of the TNFα gene when gender and hypertension, strong independent factors for stroke, were included in the model.
We analyzed the effect of hypertension in female and male stroke patients bearing the A1A1 compared to the corresponding hypertensive referents. It was revealed that hypertension does have significant effects and could predict stroke in males (p=0.037; OR=2.46), but not in females. The overall frequencies of alleles and genotypes found in stroke group and referents did not show any association to stroke.
There are no reports of the association of this polymorphic site to stroke. Our finding, that hypertension has a stronger impact on the development of stroke in male patients with A1A1 genotype, indicates that investigation in this direction should be intensified. The local regulation of the TNFα is possible different in brain tissue than in other tissues.
It can be speculated that certain haplotypes of TNF, together with other risk factors, can have an additive effects on the development on stroke, while in the case of RA, the neighboring genes also have a strong impact.

4.5. THE IL-1β TaqI POLYMORPHISM IN RA

We found no association of the alleles or genotypes of the IL-1β gene to RA. The severity of RA was associated to genotype A2A2. As in the case of the TNFα gene, the severity was presented as an accumulated disease score and patients with A2A2 had the highest score, 5.3 (p=0.02). The genotype A1A2 had an intermediate score of 4.9 and A1A1 the lowest, 4.7. This result is in the concordance with the study of Poutsiaka *et al.* (223). It was postulated that IL-1β does play an important role in the pathogenesis of RA. It might be that a "high secretory" allele A2 could lead to more severe forms of RA. There was no association to cardiovascular complications in RA patients. The allelic distribution followed the Hardy-Weinberg law.

4.6. THE IL-1β TaqI POLYMORPHISM IN STROKE

We found no association of the alleles or genotypes of the IL-1β gene polymorphism to development of stroke. No association was found when the data of stroke patients were adjusted for hypertension or gender. The allelic distribution followed the Hardy-Weinberg law.

4.7. THE IL-1Ra VNTR POLYMORPHISM IN RA

The genotype A1A2 was decreased (OR=0.52) in RA patients compared to controls, while A2A3 was increased (OR=12.11). However, the patient group was too small to be able to adequately determine the importance of genotype A2A3 in the development of RA. The biological function of the allele A3 is still unknown. Nevertheless, its frequency in our study group (OR=4.61) was significantly increased and therefore worth of mentioning.
Allele A2, previously correlated with higher secretion of the cytokine, was associated with less risk for cardiovascular complications. According to Dewberry *et al.* (224) the expression of IL-1Ra in EC is exclusively in the intracellular isoform, while in monocytes different forms of the cytokine are present. The allele A2 was associated with significantly reduced levels of IL-1Ra in human umbilical vein ECs. Thus, the same polymorphism gives different secretion pattern in different cells.

4.8. THE IL-1Ra VNTR POLYMORPHISM IN STROKE

There was no association of the IL-1Ra gene polymorphism to the development of stroke. However, when analyzing different haplotypes for IL-1β/IL-1Ra some interesting results were found.

4.9. IL-1β/IL-1Ra CROSS-GENOTYPING IN RA

Comparisons of IL-1β and IL-1Ra alleles of different haplotypes between RA patients with and without cardiovascular complication revealed that certain haplotypes were important. In RA patients with cardiovascular disease, haplotypes A1+IL-1β/A2+IL-1Ra and A2+IL-1β/A2+IL-1Ra were decreased compared to RA patients

without these complication (OR=0.20). It seemed that A2 of IL-1Ra have a protective role by blocking the action of IL-1β.

4.10. IL-1β/IL-1Ra CROSS-GENOTYPING IN STROKE

Cross-genotyping in patients who developed stroke revealed an association of the haplotype A2+IL-1β/A2+IL-1Ra (OR=3.07). In normotensive patients who developed stroke the haplotype A2+IL-1β/A2+IL-1Ra was present in 23.1%, compared to only 9% of normotensive referents.

These interesting findings in patients who developed stroke could be explained if we keep in mind that transcriptions of these cytokine genes, as well as their secretion, are differently regulated in different tissues and that different antigens are primarily inducers of either IL-1Ra or IL-1β. The high secretion of IL-1Ra in RA patients could give some protection from the development of RA, while there was no such protection for stroke patients.
The higher amount of IL-1Ra in the synovium, resulting by the presence of this high secretory allele is therefore beneficial in RA patients, but not in stroke patients. More investigation about the genetic background that could lead to development of stroke, as well as biological function of cytokines in brain should be performed.

4.11. AUTOANTIBODIES AGAINST oxLDL, MDA-LDL AND CARDIOLIPIN IN RA AND STROKE

The high mortality and morbidity in RA was previously associated to cardiovascular complications (176, 177). Several reports have associated these autoantibodies to different cardiovascular diseases, e.g. MI (78, 79), cardiac death (78), arterial and venous thrombosis (73, 225).

Paper II and III investigated the association of autoantibodies against oxidized forms of LDL and cardiolipin to RA and to development of atherosclerosis.
Papers V and VI investigated the same type of autoantibodies in patients who developed stroke.
The same referent group was used for the analysis of RA (paper II) and stroke patients (paper IV). As mentioned previously, the population from Northern Sweden is genetically different from the rest of the Swedish population. Thus, the high prevalence and the levels of autoantibodies found in our RA patients could be influenced by certain genetic backgrounds that still is unknown.

4.12. ANTIBODIES AGAINST CARDIOLIPIN IN RA

Although it is well known that RA is characterized by the presence of autoantibodies like Rheumatoid Factor (RF), ANA and aPL, the significance of these in RA is incompletely unknown.
Anticardiolipin antibodies have been suggested to be predictive markers for MI (78, 79), and arterial and venous thrombosis (73, 225). A recent prospective study of hyperlipidemic patients showed that aCL are independent risk factors for atherosclerotic vascular disease (76).

The study presented in the paper II showed that RA patients had significantly increased levels of IgG, IgM and IgA aCl ($p<0.01$) as well as increased prevalence of all three isotypes. Increased levels of IgM and IgA aCL were

found in patients with extraarticular manifestations when compared to RA patients without these complications. No association was found to vasculitic ulcers/neuropathy, pericarditis, pleuritis or scleritis.
There was no relation between the RF levels and aCL and no association with accumulated disease activity. Patients treated with corticosteroids had higher levels of IgM aCL.
Antibodies against cardiolipin seem to have a strong tendency to build up immune complexes (72, 226). The presence of CICs induces the activation of ICAM-1 and VCAM-1 (227). These molecules are very important in the process of inflammation as it was described earlier. Therefore, in the paper III we investigated also the presence of CICs in RA patients and tried to reveal the correlation of these to other inflammatory and atherothrombotic markers.
Jacobs *et al*. (228) reported that aCL of IgM isotype were present in RA patients, but no correlation was found to the severity of the disease.

The paper III is a pilot study that involved only 39 RA patients and 39 age- and sex-matched controls that were assembled from the population register of the same region. The Table 3 show the presence of established cardiovascular risk factors in patients and referents and no significant differences between groups are found.

Table 3. Established cardiovascular risk factors in 39 RA patients with a mean 23 years of disease duration.

Variable	RA N=39	Controls N=39
Hypertension, treated	9	5
Diabetes mellitus	1	0
Smoking, current or former	18	22
Cardiovascular event ever	1	1
Body mass index	22.68 (18.59-36.18)	23.74 (18.07-34.21)
sCholesterol mmol/l	5.20 (3.70-8.14)	5.80 (4.70-7.40)

One of the interesting findings is that levels of all three isotypes of aCl were significantly elevated. IgA is usually triggered by infections in intestinal or respiratory tract. IgM has the highest capacity to activate complements. It was suggested that this antibody, along with the IgG aCL, could be used as an atherothrombotic risk factor in patients with vascular disease (76). As in the paper II, RA patients had significantly higher levels of IgG, IgM and IgA aCL than respective controls. These results showed that higher levels of these autoantibodies are present in RA patients from Northern Sweden. The CIC level correlated significantly with the aCL IgM (r_s=0.49, $p<0.001$). The factor analyses that we used revealed that isotype IgM of aCL, as well as of anti-oxLDL/anti-MDA-LDL antibodies are related to CIC. The correlation of the IgM antibody cluster, CIC and the presence of atherosclerotic plaques indicate that the atherosclerosis in RA is related to IgM aCL, IgM anti-oxLDL, IgM anti-MDA-LDL and CIC.

4.13. ANTIBODIES AGAINST CARDIOLIPIN IN STROKE

In patients who developed stroke there were only marginal differences in the prevalence and concentration of aCL between the individuals that developed stroke and healthy referents (paper VI). Only IgM aCL were present in 11.4% (14/123) of those who developed stroke compared to 4.1% of healthy referents (p=0.013; OR=2.97). The levels of IgM aCl was higher (p=0.026; OR=1.34), but after the adjustment for hypertension, diabetes mellitus, current cigarette smoking and smokeless tobacco, the odds ratio decreased (OR=1.24; P= 0.077). Therefore, IgM aCL could not be considered as an independent predictive marker for stroke.

4.14. ANTIBODIES AGAINST oxLDL IN RA

The levels of all three isotypes of autoantibodies against MDA-LDL (paper II) and of IgG and IgA against oxLDL were significantly increased ($p<0.01$). Similar result were found for the IgG and IgA levels and prevalence of anti-oxLDL antibodies ($p<0.01$) (paper II). The IgM isotype of anti-MDA-LDL was increased in patients with extraarticular manifestations compared to patients without these complications. We found no association of autoantibodies against oxidized forms of LDL to cardiovascular or cerebrovascular events, such as stroke/transient ischemic attacks, hypertension and deep vein thrombosis. However, RA patients with MI had a higher prevalence of IgG anti-MDA-LDL (p=0.04) compared to patients without MI (paper II). This result was in concordance with the previous study performed in our group in which IgG anti-MDA-LDL was predictive for future MI (79). These results are first of their kind, showing that the appearance of cardiovascular complications in RA could be associated with the presence of these antibodies.

The paper III confirmed that RA patients had significantly higher levels of IgM anti-oxLDL and of IgM and IgA anti-MDA-LDL compared with referents. Also, the CIC level correlated significantly with IgM anti-oxLDL (r_s=0.36; p<0.05). Surprisingly, IgG anti-MDA-LDL showed inverse correlation with IL-6 (r_s=0.32, p<0.05), while oxLDL antibodies did not correlate with any of the measured markers of inflammation in a univariate analysis. However, IgA and IgM anti-MDA-LDL showed correlation with several markers of atherothrombogenesis, such as sICAM-1 and sE-selectin. The increased serum levels of adhesion molecules compared with controls may reflect an up-regulation of endothelial expression of adhesion molecules in RA patients. It has been shown that levels of sICAM-1 could predict CVD in healthy people (229) and this molecule can be induced in macrophages and EC by the actions of inflammatory cytokines (230). Sinisalo *et al.* (231) showed that antibodies against oxLDL are associated with vascular dysfunction and proposed that these autoantibodies should be considered as an inflammatory risk factor for coronary heart disease (231). Our study showed that sICAM-1 is significantly higher in RA patients with plaque compared to those without atherosclerotic modified vessels. The factor analysis confirmed that prolonged endothelial activation leads to the formation of plaques. Also, our findings were in concordance with the study of Aoki *et al.* (232) who found increased sICAM-1 in vasculitic RA patients.

4.15. THE CROSS REACTIVITY OF AUTOANTIBODIES AGAINST aCL, oxLDL AND MDA-LDL IN RA

In the paper III we showed that IgM aCL showed significant correlation with IgM anti-oxLDL (r_s=0.0.61, p<0.001) and with IgM anti-MDA-LDL (r_s=0.59, p<0.001). Also, the IgA aCl correlated to the IgA of anti-oxLDL and anti-MDA-LDL (r_s=0.41, p<0.05; r_s=0.36, p<0.05). Oxidized LDL antibodies of the three isotypes correlated significantly with anti-MDA-LDL antibodies (IgG: r_s=0.42, p<0.05; IgM: r_s=0.57, p<0.001; IgA: r_s=0.57, p<0.001).
It is possible that aCL recognizes the epitopes generated during a process in which lipid peroxidation and the generation of CIC are components. The presence of CIC induces the damage on the EC of the vascular wall. Probably, increased aCL and anti-oxLDL antibodies might be directed against shared antigenic epitopes. Vaarala *et al.* (65) suggested that these two are partly cross-reactive, probably due to their binding to phospholipids. There is a structural similarity between the LDL and the phospholipid-β2GPI complex, so aCL raised against this complex, might react with epitopes revealed on large molecule of oxidized LDL (99).

4.16. ANTIBODIES AGAINST oxLDL IN STROKE

There was no association of autoantibodies against oxidized forms of LDL with the development of stroke (paper V).
There are many studies that show the association of these autoantibodies to cardiovascular disease, such as carotid atherosclerosis, coronary atherosclerosis, essential hypertension and myocardial infarction (95, 96, 233, 234). However, there are others, in which the levels of these autoantibodies did nor differ in patients with non-insulin-dependent diabetes mellitus, coronary heart disease and stroke (15, 235, 236).
Stroke is a cerebrovascular disease that it is less reliable for the presence of atherosclerosis, because it includes both cerebral hemorrhage, which is mostly a result of damages achieved by hypertension and diabetes, and cerebral thrombosis.

One of the explanations that these autoantibodies were not associated to development of stroke in our cohort is that, in contrast to MI, stroke involves larger vessels. The effect of attached autoantibodies on the wall of blood vessels would in that case be less harmful.

4.17. MARKERS OF IMMUNE ACTIVATION, ENDOTHELIAL ACTIVATION AND HAEMOSTATIC FACTORS IN RA

Investigation of ESR, CRP, haptoglobin, IL-6 and IL-2sRα presented in the paper III showed that RA patients had significantly higher levels of these acute-phase reactants and of sICAM-1 and sE-selectin. These results indicate that RA patients have an evident increase of immunological markers and endothelial cell derived products. The correlations of sICAM-1 and sE-selectin with factors in hemostases, such as PAI-1, tPA and vWF (that are also products of endothelial cells) indicate that endothelial activation might lead to the atherothrombotic process in RA. Increased levels of IL-6 and IL-2sRα indicate that T-lymphocytes are involved in enhanced expression of adhesion molecules in RA. This was also supported by loading of homocysteine in the factor analysis. The homocysteine was reported to exert direct injuries on the endothelium (237).

Several factors of potential thrombogenic importance, like PAI-1 mass, vWF, D-dimer and aCL were significantly higher in RA and correlated with inflammatory marker, like ESR. Changes in the levels of haemostatic factors induce hypercoagulation and the PAI-1 thus suggested to play an important role in the formation of the plaque.
So, several atherogenic and thrombogenic factors were increased in RA patients resulting in the continuos activation of the endothelium and acceleration of atherosclerosis.

5. CONCLUSIONS AND FUTURE STUDIES

Study of polymorphisms of pro-inflammatory cytokines IL-1β, IL-1Ra and TNFα revealed that these polymorphic sites are of importance and should be investigated in more details in both RA and especially in stroke.

In RA patients, we found a strong association of allele A1 of TNFα to RA and an association of A2 allele to more severe forms of the disease. The genotype A1A2 of IL-1Ra was decreased in this group and the "high secretory" allele A2 of this anti-inflammatory cytokine decreased the risk for cardiovascular complications. Different polymorphic sites of IL-1β and IL-1Ra should be analyzed and correlate to different HLA haplotypes.

Today's treatment of RA involves the use of both classical treatments with corticosteroids or non-steroidal, anti-inflammatory drugs (NSAID) and new treatments with blockage of pro-inflammatory cytokines. As shown in the study of Ulfgren *et al.* (238), there are both interindividual and intra-articular variations of pro-inflammatory cytokines. These finding, together with ours, imply that patients selected for the treatment with cytokine blockade should be investigated for the presence of certain "high secretory" and certain haplotypes.

The study of patients who developed stroke showed association of A1A1 of TNFα to the development of stroke in hypertensive male patients. There was no association of IL-1β or IL-1Ra polymorphisms. The haplotype A2+ IL-1β/A2+ IL-1Ra was increased in normotensive stroke patients, indicating that allele A2 of IL-1Ra might have even in this group some protective function. This result, as well as functional implication, should be investigated in more detail.

Future studies should include typing for HLA DR and DQ haplotypes in addition to the polymorphisms of pro-inflammatory cytokines. In addition, studies of polymorphisms in RA patients treated with cytokine blockade treatment are started. It would be interesting to see up how patients of different haplotypes respond to this treatment.

Increased levels of autoantibodies against cardiolipin and oxLDL were present in RA patients. Increased levels of IgM and IgA aCL were found in patients with extraarticular manifestations when compared to RA patients without these complications. It was also presented that IgM of aCL correlated with CIC and antibodies against oxidized forms of LDL. Antibodies of IgM isotype, in the form of CICs correlated to the formation of atherosclerotic plaques in RA patients.

Antibodies against oxLDL and MDA-LDL were significantly increased in RA patients. Of significance is that IgM anti-MDA-LDL was significantly increased in RA patients with extraarticular manifestations. We showed in paper II that the prevalence of IgG anti-MDA-LDL was increased in MI patients. This result was in concordance with a previous study performed in our group, in which IgG anti-MDA-LDL was predictive for future MI (79). In addition, IgA and IgM anti-MDA-LDL showed correlation with several markers of atherothrombogenesis. These results suggest that antibodies against MDA-LDL might have a significant function in the pathogenesis of atherosclerosis of RA patients. Therefore, the screening of RA patients for the presence of these antibodies in early phases of the disease should be performed. The purpose of this would be to recognize the risk for severe forms of the disease and cardiovascular complications and to allow for prevented treatment.

There were only marginal differences in the prevalence and concentration of aCL between the patients who developed stroke and referents. Study of oxLDL autoantibodies did not revealed any association of these antibodies to the development of stroke. Thus, these antibodies do not seem to be of predictive values for stroke.

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