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LIPIDS AND AUTOIMMUNITY IN ATHEROSCLEROSIS AND CARDIOVASCULAR DISEASE

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Abstract

Atherosclerosis is an inflammatory disease. The rupture of atherosclerotic plaques is the major cause of severe cardiovascular disease (CVD), such as myocardial infarction (MI) and stroke. The stability of the plaque depends on its cellular and extracellular contents. Plaques with large lipid cores, thin fibrous caps, and active inflammation are most likely to rupture. Oxidized low density lipoprotein (oxLDL) is proinflammatory and can promote both apoptosis and necrosis at higher concentrations and could play important roles in promoting plaque rupture. We and others have demonstrated that inflammatory phospholipids such as platelet activating factor (PAF)-like lipids and lysophosphatidylcholine (LPC) could be of major importance in oxLDL. Antibodies against phosphocholine exposed on inflammatory phospholipids in oxLDL, are risk factors for CVD at low levels, though the mechanisms by which anti-PC protect have not been clear. Another factor that we have proposed to interfere with inflammatory phospholipids is the protein Annexin A5. Incidence of CVD in systemic lupus erythematosus (SLE) patients is exceedingly high, and is an important clinical problem. Further, this renders SLE an interesting “human model” for CVD and especially for the study of the role of immune reactions and autoimmunity in CVD.

Aim. The objective of this study is to investigate by what mechanisms of anti-PC may protect against CVD; the role of inflammatory phospholipids like LPC and others including cardiolipin (CL) in atherosclerosis and CVD and if Annexin A5 (ANXA5), has any potential therapeutic role. In addition, the roles of dyslipidemia and anti-PC in SLE are also investigated.

In **Paper I**, the underlying mechanisms of anti-PC and SLE-related CVD were studied. The study group consisted of 26 women with SLE with a history of CVD (myocardial infarction, angina pectoris, thromboembolic stroke or intermittent claudication), 26 age-matched women with SLE but without clinical manifestations of CVD and 26 age-matched control women. IgG anti-PC were decreased among the SLE-cases and SLE-controls as compared to the controls, respectively. SLE cases were more prevalent in the lowest 25th percentile of anti-PC-IgM (and IgG) as compared to the controls. Among the SLE cases, anti-PC were associated negatively with disease severity. We could extract IgG anti-PC from human immunoglobulin (Ig) and the specificity of aPC-IgG was confirmed by a competition ELISA. PAF-induced expression of adhesion molecules by HUVECs could be inhibited by these IgG anti-PC which suggests a mechanism by which anti-PC could be atheroprotective. In conclusion, we report that there were low levels of anti-PC in SLE-patients (where antibody production is typically very high) and demonstrate that anti-PC can be extracted from human Ig. Also, these anti-PC have anti-inflammatory properties with direct relevance to CVD.

In **Paper II**, we focused on CL and could demonstrate that this phospholipid could be oxidized in vitro with results confirmed by mass spectrophotometry. In contrast to native CL, oxCL can significantly induce ICAM-1 and VCAM-1 expression on HUVECs; activate intracellular calcium mobilization and induce production of leukotrienes (LTB₄). These effects of oxCL were inhibited by ANXA5, which bound oxCL. In conclusion, we demonstrate for the first time that oxCL but not native CL can cause inflammatory effects that could contribute to human chronic inflammatory disease in general and CVD. ANXA5 could have potential therapeutic role.

In **Paper III**, we studied the pro-inflammatory effects of oxLDL and LPC and how ANXA5 interfere with these, with relevance to CVD and plaque rupture. Both oxLDL and LPC stimulated the expression of MMP-9 in co-cultures of HUVECs and macrophages and LPC induced LTB₄ production in macrophages, effects which could be inhibited by ANXA5. Furthermore, ANXA5 was capable of inhibiting macrophage uptake of oxLDL in a concentration dependent manner. These results support the notion that oxLDL and LPC are involved in CVD and plaque rupture. ANXA5 could play a role as a novel anti-inflammatory agent in atherosclerosis and CVD and inhibit foam cell formation.

In **Paper IV**, the focus was placed on dyslipidemia in SLE. Small dense LDL (sdLDL), usually considered proatherogenic, were not raised among SLE and SLE-related CVD. The lipoprotein pattern in SLE and SLE-related CVD was not typically proatherogenic. Small HDL often assumed to be less atheroprotective than larger HDL-particles, were also found less common among both SLE cases and SLE controls. LDL from SLE patients did not bind to proteoglycans more than LDL from controls. The lipid profile in SLE-related CVD does not appear to be typically atherogenic.

Conclusions: oxLDL, LPC and oxCL can promote the production of factors implicated in inflammation and plaque rupture, and these effects can be inhibited by ANXA5. Both SLE cases and SLE controls exhibited low anti-PC levels and this might contribute both to CVD in SLE and to SLE per se. Anti-PC extracted from IVIG can neutralize PAF-induced inflammatory effects, suggesting a potential atheroprotective role for anti-PC.

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有志者事竟成

LIST OF PUBLICATIONS

- I. Jun Su*, **Xiang Hua***, Hernan Concha, Elisabet Svenungsson, Anna Frostegård and Johan Frostegård. Natural antibodies against phosphorylcholine as potential protective factors in SLE. *Rheumatology* 2008 47(8):1144-1150. (*Contributed equally)

- II. **Xiang Hua**, Min Wan, Jun Su, Anna Frostegård, Jesper Haeggström and Johan Frostegård. Oxidized cardiolipin has pro-inflammatory effects which are inhibited by ANXA5: implications for cardiovascular disease and chronic inflammation. (Submitted)

- III. Helena Domeij*, **Xiang Hua***, Jun Su, Anna Frostegård, Jesper Haeggström, Tomas Modéer and Johan Frostegård. ANXA5 inhibits atherogenic and proinflammatory effects of oxidized LDL and lysophosphatidylcholine: implications for plaque rupture and cardiovascular disease. (Manuscript, *Contributed equally)

- IV. **Xiang Hua**, Jun Su, Elisabet Svenungsson, Eva Hurt-Camejo, Kerstin Jensen-Urstad, Bo Angelin, Peter Båvenholm and Johan Frostegård. Dyslipidemia and lipoprotein pattern in SLE and SLE-related cardiovascular disease. *Scandinavian Journal of Rheumatology*, 2009 Jan 22:1-6.

LIST OF ABBREVIATIONS

ANXA5	Annexin A5
apoB	apolipoprotein B
anti-CL	anticardiolipin antibodies
anti-PC	anti- phosphorylcholine antibodies
AA	arachidonic acid
aApo A-1	autoantibodies against apolipoprotein A1
aLPL	autoantibodies against lipoprotein lipase
CL	cardiolipin
CVD	cardiovascular disease
CEs	cholesteryl esters
CETP	cholesterol ester transfer protein
CM	chylomicrons
CRP	c reactive protein
CHD	coronary heart disease
cPLA2	cytosolic PLA2
DCs	dendritic cells
DMSO	dimethyl sulfoxide
ELISA	enzyme-linked immunosorbent assay
ECM	extracellular matrix
5-HPETE	5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid
5-LO	5-lipoxygenase
FLAP	5LO activating protein
GPCR	G protein coupled receptors
GM-CSF	granulocyte-macrophage colony stimulating factor
HF	heart failure
HDL	high density lipoprotein
HUVECs	human umbilical vein endothelial cells
ICAM-1	Intercellular adhesion molecule-1
IL-1 β	interleukin-1 β
IMT	intima-media thickness
IVIG	intravenous immunoglobulin
IVUS	Intravascular ultrasound
LCAT	lecithin: cholesterol acyltransferase
LTA ₄	leukotriene A4
LTB ₄	leukotriene B4
LPL	lipoprotein lipase
LDL	low-density lipoprotein
LDLR	LDL receptor
LPC	lysophosphatidylcholine
MRI	magnetic resonance imaging
MMPs	Matrix metalloproteinases
MCP-1	monocyte chemoattractant protein-1
MI	myocardial infarction
NMR	nuclear magnetic resonance

Lipids and autoimmunity in atherosclerosis and cardiovascular diseases

NADH	nicotinamide adenine dinucleotide dehydrogenase
NSAIDs	non-steroidal anti-inflammatory drugs
NF- κ B	nuclear factor κ B
oxLDL	oxidized low density lipoprotein
oxPL	oxidized phospholipids
POVPC	1-palmitoyl-2-oxoaleroyl-sn-glycero-3-phosphorylcholine
PAMPs	pathogen-associated molecular patterns
PtdOH	phosphatidic acid
PTC	phosphatidylcholine
PE	phosphatidylethanolamine
PtdGro	phosphatidylglycerol
PS	phosphatidylserine
PLA2	phospholipase A2
RTX	rituximab
PC	phosphorylcholine
PAF	platelet activating factor
PECAM-1	platelet endothelial cell adhesion molecule-1
PROCAM	The prospective Cardiovascular Study in Munster
SRS-A	slow Reacting Substance of Anaphylaxis
SM	sphingomyelin
sdLDL	small dense LDL
SLE	systemic lupus erythematosus
TIMPs	tissue inhibitors of metalloproteinases
TGF β	transforming growth factor β
TG	triglyceride
VCAM-1	vascular cell adhesion molecule-1
VLDL	very low density lipoptoetin

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Introduction

Cardiovascular disease (CVD) remains the leading cause of morbidity and mortality in the Western world and it is increasing rapidly in the developing countries, and will soon become a pre-eminent health problem worldwide. If these trends will continue in the early part of 21st century (Genest 2000). With atherosclerosis as one of the major causes, the search of the pathogenesis and potential mechanisms involved in atherosclerosis, have become important targets for clinicians and medical researchers. Several big epidemiological studies have been successfully conducted, one of which being the Framingham heart study. This study began in 1949 and was designed to “seek a single essential cause to CVD” (Kannel, Dawber et al. 1961). However, they soon realized it is not the case. Multifactorial interactions may have caused the pathogenesis of atherosclerosis and CVD. Traditional risk factors have now been identified, based on large prospective cohort studies (Kannel, Dawber et al. 1961; Keys, Menotti et al. 1984). These major risk factors include high blood pressure, dyslipidemia, aging, smoking and diabetes mellitus. There is evidence supporting the idea that controlling such risk factors can reduce morbidity and mortality of CVD in patients. Nevertheless, these traditional risk factors may still fail to predict the occurrence of CVD in populations. Some CVD patients do not even have these traditional risk factors. In the last 20 years, non-traditional risk factors (Hartvigsen, Chou et al. 2008) and new pathogenesis theories (Karnoutsos, Papastergiou et al. 2008) have emerged and gained wide acceptance in the field of cardiovascular research, especially inflammatory factors and lipid oxidative theory, with both having acquired great attention (Jonasson, Holm et al. 1986; Hansson 1993; Witztum 1994; Frostegård, Ulfgren et al. 1999; Ross 1999). Our major focus is on the study of lipids and natural immunity in atherosclerosis and CVD. Based on our previous findings, an EU CVDimmune project has been assigned and is in progress both in our group and also among our collaborators. The purpose of this thesis is to discuss the effects of lipids and autoimmunity in the development of atherosclerosis, CVD.

Atherosclerosis

Atherosclerosis is a chronic disease which may begin as early as in fetal life and progresses in childhood and adolescence, contributing to the burden of CVD. Much progress has been made in understanding the development of atherosclerosis.

Plaque Structure

Intima, the innermost layer of an artery, consists of loose connective tissues, covered by a monolayer of endothelium. It is already known that early atherosclerosis consists of fatty streak which develops in the intima. Fatty streaks, though not clinically significant, are the precursors of more advanced lesions. The streaks consist of subendothelial accumulations of cholesterol-engorged macrophages, known as 'foam cells'. Fatty streaks can progress into mature atherosclerotic plaques with the accumulation of additional inflammatory-cell subsets and extracellular lipids (Skalen, Gustafsson et al. 2002; Binder, Horkko et al. 2003). The core region is surrounded by a cap of smooth muscle cells and a collagen-rich matrix. Fatty streaks in humans develop early in childhood. Lesions usually exist during teenage years, in the aorta, in the coronary arteries in the second decade, and in the cerebral arteries in the third or fourth decades (Lusis 2000). Cells in the plaque secrete different cytokines and growth factors, which together with the further deposition of extracellular matrix components contribute to the progression of plaques which results in narrowing of the arterial lumen (stenosis). The stability of the advanced atherosclerotic lesions or plaques depends on their cellular and extracellular contents. Plaques are usually stable with small lipid cores, thick fibrous caps, few inflammatory cells and a preponderance of smooth muscle cells; conversely, those with large lipid cores, thin fibrous caps, numerous macrophages and relatively few smooth muscle cells are most likely to rupture (14). Calcification, ulceration at the luminal surface, and haemorrhage from small vessels render plaques a high risk of rupture.

There are preferred sites of lesion formation within the arteries because of differences in blood flow dynamics. Plaque rupture usually occurs at the base of the fibrous cap, which is usually thin and contains relatively few smooth muscle cells but many inflammatory cells. When the plaques rupture, platelets and coagulation factors in circulating blood are exposed to the thrombogenic contents of the plaques' extracellular matrix and lipid core,

including collagen, phospholipids and tissue factor. Thereafter, the disrupted plaque serves as a scaffold to allow platelet aggregation and coagulation. The production of thrombin, subsequent conversion of fibrinogen to fibrin, and release of von Willebrand factor from activated platelets create a cross-linked network that allows the formation of a thrombus. The thrombus size depends on the extent of plaque rupture as well as the activity of the endogenous fibrinolytic pathway. When sufficiently large, the thrombus can either partially or completely occlude the coronary vessel lumen and cause an acute coronary event (Zaman, Helft et al. 2000; Libby and Theroux 2005; Toth 2008; Wagner and Frenette 2008).

Cellular composition in the atherosclerotic plaque

Substantial evidence now indicate that a wide range of cellular effectors promote atherogenesis by mediating inflammatory cascades.

Unexpected roles for neutrophils in atherogenesis were recently discovered. Lipid-scavenging macrophages and dendritic cells (DCs) interact with T cells and contribute to plaque progression. In addition, a heterogeneous role for adaptive immune-cell subsets in atherogenesis is illustrated by the participation of TH2 cells, regulatory T cells and B-1 cells, which seem to have atheroprotective roles (Lusis 2000). In this thesis, I focused on the role of macrophages, endothelial cells and neutrophils (Figure 1).

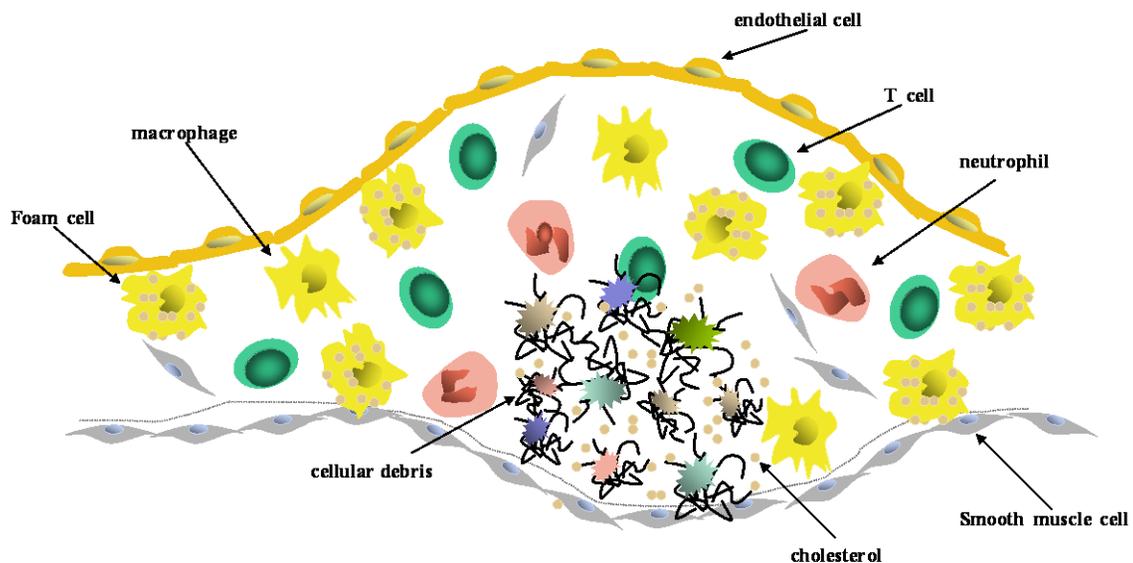


Figure 1. Cellular components in plaque

Macrophages

Monocytes differentiate into macrophages in the intima and become loaded with cell-activating oxidized low density lipoproteins (oxLDL) and other lipids. Subsequently, they accumulate in the involving lesion and transform into foam cells giving rise to early plaques (known as fatty streaks) in the intima. Internalization of modified low-density lipoproteins (LDL) via macrophage scavenger receptors (e.g. scavenger receptor A and CD36) is thought to play a crucial role in the development of atherosclerotic lesions. Cilostazol, an antiplatelet agent with selective phosphodiesterase 3 inhibitory action, can decrease scavenger receptor A, significantly inhibited foam cell formation and ameliorate atherosclerosis in mouse models (Okutsu, Yoshikawa et al. 2008). In line with this, there was a 76.5% decrease in aortic tree lesion area (Western diet) and a 45% decrease in aortic sinus lesion area (normal chow) in the CD36-apo E double-null mice as compared to controls (Febbraio, Podrez et al. 2000). Inhibition of the scavenger function is generally believed to be atheroprotective.

Endothelial cells

Endothelial lining presents a large surface area for the exchange of materials between blood and tissues. The total area of this interface in human has been estimated to be about 350m² (Pries, Secomb et al. 2000). The intimal surface of healthy endothelium is both anticoagulant and anti-thrombotic. In the quiescent state, endothelial cells maintain blood fluidity by promoting the activity of numerous anticoagulant pathways, the most important of which being the protein C/protein S pathway. Activated protein C inactivates two cofactors which are essential for blood coagulation: factors VIIIa and Va. Vessel damage or exposure to certain cytokines or proinflammatory stimuli shifts the balance towards a procoagulant/prothrombotic phenotype of the endothelial cells (Figure 2) (Carine 2003) .

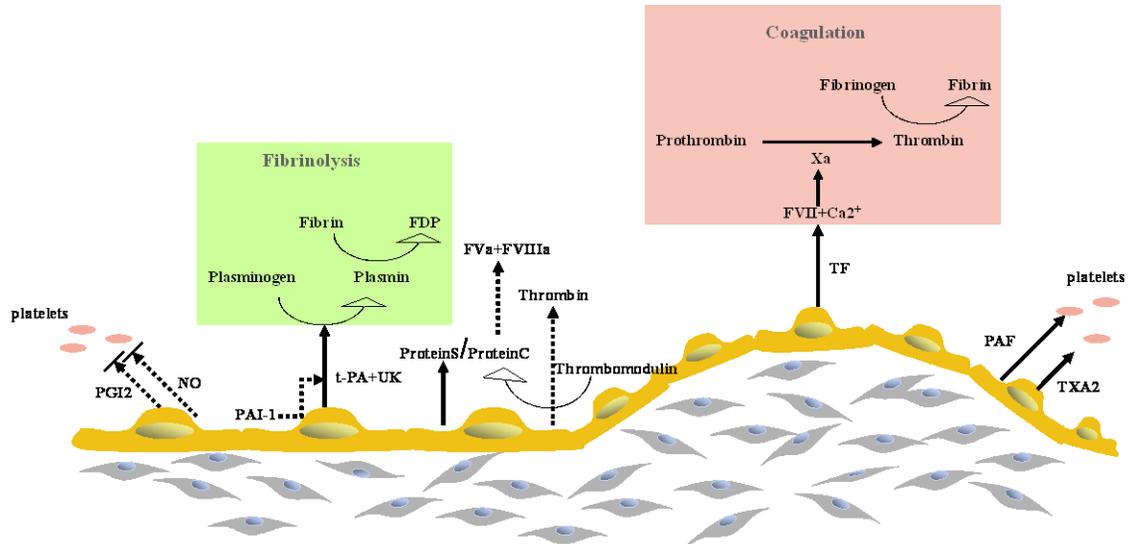


Figure 2. Shift to pro-coagulant and thrombotic when endothelial cells become activated.
 Modified from M. Varine. 2003

Endothelial cells are sensitive to damage. Endothelial cell injury is responsible for the localization of the cellular elements that drive the development of atherosclerosis. In 1973 Ross and Glomset proposed that atherosclerosis results from arterial responses to chronic injury, and changes in the injured endothelium disrupt its permeability characteristics, thus permitting the interaction between elements of the blood and the arterial wall. Biochemical signals within the endothelium downregulate protective mechanisms and upregulate the synthesis of proteins that recruit platelets, monocytes, and lymphocytes, resulting in the excessive inflammatory and fibroproliferative responses (Ross and Glomset 1973).

One of the earliest cellular responses in the formation of atherosclerotic lesions is leukocyte adherence to the endothelium in the artery wall, a process which involves multiple steps (Gerrity 1981). The first step is to allow circulating cells to tether and roll along the vessel wall, which is transient and primary. In this phase, endothelial P- and E-selectins bind to carbohydrate ligands on leukocytes; and leukocyte integrin very late – acting antigen-4 interacts with vascular cell adhesion molecule-1 (VCAM-1) expressed on endothelial cells. The second step involves chemoattractant release from tissues and

the expression on endothelial cells of intercellular adhesion molecule-1 (ICAM-1) and VCAM-1 which mediate firm adhesion. The final step involves chemotactic transmigration and the expression of Platelet endothelial cell adhesion molecule-1 (PECAM-1) which is required for monocytes to migrate across the endothelial lining into the underlying tissue (Nakashima, Raines et al. 1998; Mestas and Ley 2008; Laufer, Winkens et al. 2009).

Neutrophils

Neutrophils are among the first cells to respond to invading microorganisms or tissue damage. They can endocytose “foreigners” and producing reactive oxygen species, myeloperoxidase and various proteolytic enzymes, such as elastase, which together function to eliminate microbial pathogens. At the same time, however it can also contribute to tissue destruction. There is evidence suggesting that neutrophils also contribute to the development of CVD. Histological analysis of plaques in cerebral arteries suggests the involvement of neutrophils in late-stage plaques. Another evidence of neutrophils involvement in apoE deficient mice is the presence of neutrophils mainly in the luminal-plaque regions and also in the adventitia. In addition, neutrophils accumulate in the arterial adventitia during atherogenesis in LDL receptor (LDLR)-deficient mice. Finally, adoptively transferred neutrophils were recruited from the blood to the lesions, indicating that they continually traffic to chronically inflamed arteries, where they secrete pro-inflammatory mediators and can promote plaque growth and instability. Evidences show that neutrophils can produce leukotrienes under stimulation, which contributes to plaque instability.

Early diagnosis of atherosclerosis

A variety of invasive and non-invasive techniques are available to measure atherosclerosis and subclinical atherosclerosis.

Non-invasive techniques

Non-invasive techniques like B-mode ultrasonography can determine the combined thickness of the arterial intimal and medial layers, usually measured in the common carotid artery (Handa, Matsumoto et al. 1990). The carotid intima-media thickness (IMT)

reflects the diffuse thickening of the intimal layer seen in atherosclerosis and has been validated as a measure of the risk for cardiovascular events and atherosclerotic disease burden (Salonen and Salonen 1993; Simon, Gariepy et al. 2002).

Risk Score, Baldassarre et al. studied 1969 dyslipidaemic patients considered to be at low or intermediate risk who underwent carotid ultrasound at a lipid clinic. Both Framingham Risk Score (Wilson, D'Agostino et al. 1998) and carotid IMT were independent predictors of outcome ($p < 0.04$ for both), and the measurement of IMT significantly improved the predictive value of the Framingham Risk Score ($p = 0.04$). Patients whose Framingham scores placed them at intermediate risk were determined to be at high risk when their carotid IMT was above the 60th percentile (men) or the 80th percentile (women) of the maximum IMT distribution (Baldassarre, Amato et al. 2007).

High resolution magnetic resonance imaging (MRI) is also used to evaluate plaque volume and composition, fibrous cap integrity, and lesion type plaque burden and susceptibility to rupture.

Coronary calcium level is a reflection of plaque burden. because calcium deposits are related to the lipid and apoptotic remnants of the plaque, electron-beam Computed tomography CT, which is used clinically, measures coronary artery calcification (Toth 2008).

Magnetic resonance has been applied to measure lipoprotein profile. Traditionally, the cholesterol content of LDL and high density lipoprotein (HDL) particles (LDL-C and HDL-C) have been measured but results vary widely because lipoprotein can differ in their cholesterol and triglyceride (TG) content. LDL numbers can be quite different despite having the same concentration of LDL-C; therefore the risk of CVD development can be different as well. The technique of magnetic resonance has been applied in clinics based on the knowledge that each lipoprotein subclass particle emits distinctive nuclear magnetic resonance (NMR) signals. Subclass signal amplitudes are directly proportional to the particle numbers, irrespective of variation in particle lipid composition.

Invasive techniques

Coronary angiography can clinically be applied to localize plaques and reveal the degree of coronary luminal stenosis. However, angiography cannot predict the time and occlusion location (Little, Constantinescu et al. 1988).

Intravascular ultrasound (IVUS), combined with contrast agents can be used to quantify the size and composition of plaques along the entire thickness of the vessel wall and provide information about lesion location and the magnitude of plaque burden. IVUS can also identify enlarged plaques that are not yet infringing on the arterial lumen (DeMaria, Narula et al. 2006; Hansson and Libby 2006).

Lipoproteins and Lipids

Lipoproteins are complexes of lipids with proteins

Lipoproteins are multicomponent complexes of proteins and lipids. Each type of lipoprotein has a characteristic molecular mass, size, chemical composition, density, and physiological role. The protein and lipid in the complex are held together by noncovalent forces.

Plasma lipoproteins are extensively characterized and changes in their relative amounts are predictive of atherosclerosis. They assume many roles in blood, including the transportation of lipids from tissue to tissue and participation in lipid metabolism. Lipoprotein classes are categorized by their density, as determined by ultracentrifugation and electrophoresis. The protein components of a lipoprotein particle are the apolipoproteins (Sjöberg, Su et al.).

According to the difference in particle buoyant density, lipoproteins are classified into chylomicrons (CM), very low density lipoproteins (VLDL), LDL and HDL. CM are assembled in the intestinal mucosa and leave the intestine via the lymphatic system to enter into the circulation. CM transport dietary triacylglycerols to adipose tissue and muscle and dietary cholesterol to the liver. The apoC-II in the CM activates lipoprotein lipase (LPL) in the presence of phospholipids. In the capillaries of adipose tissues and muscles, the fatty acids of CM are removed from the triacylglycerols by the action of LPL. During the removal of fatty acids, a substantial portion of phospholipids is transferred to HDLs (apoA and apoC) (Mathews C 1999).

The key role played by low-density lipoprotein (LDL) particles in the pathogenesis of coronary heart disease (CHD) has been well accepted (Saad, Virella et al. 2006). Small

dense LDL has been suggested to be more atherogenic than larger-sized LDL due to its increased ability to penetrate the arterial wall, lower affinity to LDL receptor, prolonged half time in plasma, and greater propensity to be oxidized as compared with large LDL (Berneis and Krauss 2002; Koba, Yokota et al. 2008).

VLDLs are the molecules which transport endogenously derived triacylglycerols to extra-hepatic tissues. Triacylglycerols are packaged into VLDLs and released into the circulation for delivery to the various tissues (primarily muscle and adipose tissues) for storage or production of energy through oxidation. LDLs are the primary plasma carriers of cholesterol, delivering it to all tissues (cholesterol synthesized by the liver can be transported to extra-hepatic tissues if packaged in VLDLs). The uptake of LDLs occurs predominantly in the liver (75%), adrenals and adipose tissues (Figure 3)(Shalet 2002).

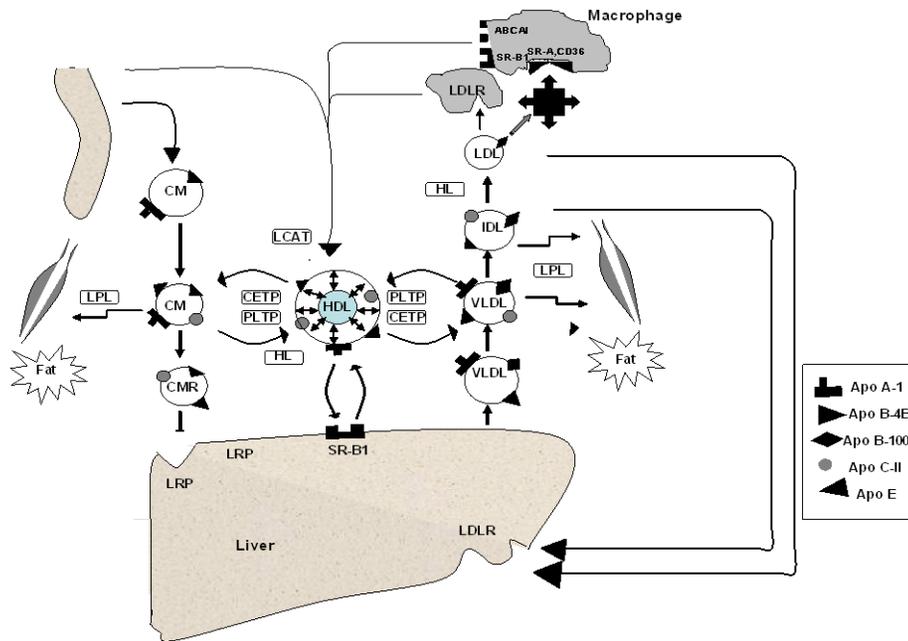


Figure 3. Lipoprotein synthesis and metabolism

Adapted from B. Angelin 2002

HDLs are synthesized de novo in the liver and small intestine, primarily as protein-rich disc-shaped particles. These newly formed HDLs are nearly devoid of any cholesterol or cholesteryl esters. Free cholesterol present in CM remnants and VLDL remnants (IDLs) can be esterified through the action of the HDL-associated enzyme, lecithin cholesterol acyltransferase, LCAT. Cholesterol-rich HDLs return to the liver, where they are endocytosed. Hepatic uptake of HDLs, or reverse cholesterol transport, may be mediated

through a HDL-specific apoA-I receptor or through lipid-lipid interactions. Macrophages also take up HDLs through apoA-I receptor interaction. HDLs can therefore acquire cholesterol and apoE from the macrophages, and the cholesterol-enriched HDLs are then secreted from the macrophages. The cholesterol esters of HDLs can also be transferred to VLDLs and LDLs through the action of the HDL-associated enzyme cholesterol ester transfer protein (CETP). This allows the excess cellular cholesterol to be returned to the liver through the LDL-receptor pathway as well as the HDL-receptor pathway.

HDL is believed to confer protection against atherosclerosis primarily by mediating reverse cholesterol transport from peripheral tissues (e.g., macrophages in the artery wall) to the liver (Genest 2000; Trigatti, Rigotti et al. 2000). It also exerts anti-oxidative, anti-thrombotic, and anti-inflammatory effects (Watson, Berliner et al. 1995; Naqvi, Shah et al. 1999). In the setting of inflammation, HDL can become pro-inflammatory under acute phase response. The paraoxonase activity is greatly inhibited and HDL can no longer prevent the oxidation of LDL but can itself become proinflammatory (Van Lenten, Hama et al. 1995; Chung, Oeser et al. 2008).

The prospective Cardiovascular Study in Munster (PROCAM) found that a high ratio of LDL-C to HDL-C increased cardiovascular risks by five fold; and in the presence of mild to normal hypertriglyceridemia it increased risks by 10-fold (Assmann, Schulte et al. 1996).

CETP and CVD

CETP can bind to lipoprotein surface and transfer cholesterol and triglyceride among LDL, VLDL and HDL. The role of CETP is highlighted from the discovery that CETP deficiency is the main cause of high HDL-C levels in Asian populations and the presence of CETP inhibitors can effectively increase HDL-C concentrations. CETP promotes the transfer of cholesteryl esters (CEs) from HDL to apolipoprotein B (apoB)-containing particles, like VLDL and LDL, in exchange for triacylglycerols. Rabbits which exhibit a high activity of CETP are susceptible to atherosclerosis. Rodents, naturally CETP-deficient, are relatively resistant to atherosclerosis (Foger, Ritsch et al. 1996). However the relationship between reduced CETP and the susceptibility to cardiovascular diseases

is complex in humans, as exemplified by the work of Hirano and coworkers, which found a reduced activity of CETP is associated with susceptibility to atherosclerosis (Hirano, Yamashita et al. 1995).

Phospholipids and CVD

Lipids also comprise of molecules such as fatty acids and their derivatives (including tri-, di-, and monoglycerides and phospholipids), as well as other sterol-containing metabolites such as cholesterol (Fahy, Subramaniam et al. 2005). The main biological functions of lipids include energy storage, serving as structural components of cell membranes, and acting as important signaling molecules (Huang 1999; Marathe, Harrison et al. 2000; Pegorier, Stengel et al. 2006).

Phospholipids exist both in lipoproteins and cell membranes. Oxidized phospholipids (oxPL) are generated when LDL or cellular phospholipids containing polyunsaturated acids undergo oxidative reaction. oxPL can be modified by serum phospholipase A2 (PLA2), paraoxonase, Nicotinamide adenine dinucleotide Dehydrogenase (NADH) oxidase, lipoxygenase, myeloperoxidase, platelet-activating factor (PAF) and acetylhydrolase. oxPL has been shown to play important roles in fatty streak formation as well as monocyte/endothelial interaction. LPC can prevent vascular relaxation.

There are considerable evidences demonstrating that phospholipid modification of lipoproteins will result in their uptake by CD36. It has been suggested that 1-palmitoyl-2-oxoaleroyl-sn-glycero-3-phosphorylcholine (POVPC) binds to both CD36 and SRA-1. In addition, activation of G protein coupled receptors (GPCR), through the Gs pathway by oxPL has also been shown. However, some oxidized phospholipids, like POVPC has been demonstrated to coneract the lipolysaccharide (LPS) pathway by binding to CD14 and LPS-binding protein. Henceforth, inhibiting nuclear factor- κ B (NF- κ B) and suppressing inflammatory reactions. These differences are related to oxPL's ability to induce chronic inflammation in the artery wall but on the other hand, participating in the negative feedback mechanism triggered by an acute Gram-negative induced inflammation.

CL and its oxidative derivatives

Cardiolipin (CL) was first isolated from beef heart by Pangborn (1942), who demonstrated that this substance was essential for the reactivity of beef heart antigens in a serological test for syphilis (LeCocq and Ballou 1964).

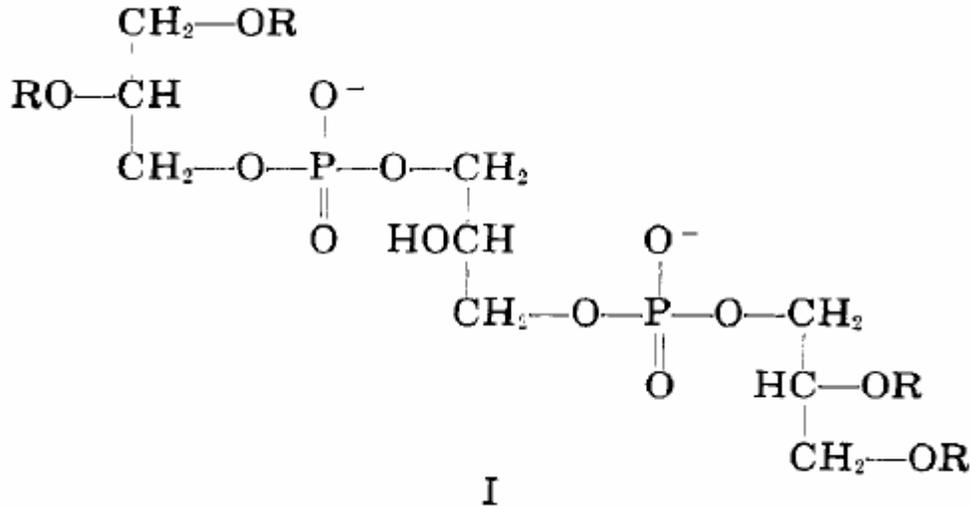


Figure 4 Structure of cardiolipin.

The name “cardiolipin” alludes to the tissue from which it was first isolated in 1942. Indeed, CL is most abundant in the mammalian hearts and to this day commercial preparations of CL are derived from heart tissues. However, CL can be found in all mammalian tissues and throughout the eukaryotic kingdom wherever mitochondria is present.

CL is a dimeric phospholipid in which two phosphatidyl moieties are linked by a central glycerol group (Figure 4). Treatment with phospholipase D yields two phospholipid products, namely phosphatidic acid (PtdOH) and phosphatidylglycerol (PtdGro). Accordingly, cardiolipin can be chemically synthesized from PtdOH and PtdGro.

CL is a unique phospholipid with a structure enriched by oxidation-sensitive linoleic acid groups that are susceptible to be oxidation (Schlame, Rua et al. 2000; Chicco and Sparagna 2007). Initially it was thought to be found exclusively in bacteria and in the inner mitochondrial membrane and are designed to simply generate an electrochemical

potential for substrate transport and ATP synthesis. However, it was not recognized as a significant physiologic plasma component (Basova, Kurnikov et al. 2007; Belikova, Jiang et al. 2007).

Accumulating evidence now suggest that this unique lipid peroxidation favors the delocalization of cytochrome c and predisposes its release from mitochondria, thus leading to the activation of the cell death programmes (Nakagawa 2004; Chicco and Sparagna 2007; Gonzalvez and Gottlieb 2007). Phospholipids found in plasma lipoproteins include phosphatidylcholine (PTC), lysoPC, phosphatidylethanolamine (PE), sphingomyelin (SM), phosphatidylserine (PS), and phosphatidylinositol. Until recently it was discovered that CL is a component of the human plasma lipoprotein, especially in LDL (Deguchi, Fernandez et al. 2000).

Previously, it has been proven that CL is easily oxidized in vivo and in vitro. Recently, oxCL has also been found in abundance in apoptotic cell membranes. Similar to phosphorycholine, oxCL belongs to a novel class of pathogen-associated molecular patterns (PAMPs) (Tuominen, Miller et al. 2006), which suggests probably novel roles for oxCL in vivo. The presence of oxCL on the apoptotic cell membranes might be crucial for natural antibodies to clear apoptotic bodies, which failure to do so might be related to autoimmune diseases. In systemic erythmatosus lupus (SLE) patients, there is a large quantity of apoptotic bodies which triggers the production of autoantibodies and the oxidation of CL; both of which can posses interesting roles.

A new finding is that oxCL is present in atherosclerotic plaques, which suggests potential pathological roles of oxCL in the development of atherosclerosis and cardiovascular disease. Monoclonal IgM antibodies specific to oxCL (LRO1) was cloned from a nonimmunized LDLR^{-/-} mouse. LRO1 has the capacity to bind to oxCL, oxLDL, apoptotic cells, and atherosclerotic lesions (Tuominen, Miller et al. 2006) which suggests that oxidation products from CL might be important in the development of cardiovascular disease.

Anticardiolipin antibodies

Much progress has been made on the study of anticardiolipin antibodies (anti-CL). The term “anticardiolipin antibodies” refers to antibodies that produce a positive response in a

specific enzyme-linked immunosorbent assay using solid-phase CL as the antigen. These antibodies are a diagnostic hallmark of SLE and primary antiphospholipid syndrome which are characterized by thrombocytopenia, recurrent thrombosis, and repeated fetal loss. However, anti-CL are also associated with syphilis and infections in general, as well as rheumatoid arthritis, psoriatic arthritis, Sjögren's syndrome, and some neurological disorders (Lazarevic, Vitic et al. 1993; Tincani, Allegri et al. 2001; Pierangeli and Harris 2008).

anti-CL are associated with increased risk of thrombosis in humans (Tincani, Allegri et al. 2001); in animals, anti-CL antibodies have also been shown to induce thrombosis. Although the thrombogenic mechanisms of anti-CL antibodies are unproven and the roles of CL in coagulation reactions are uncertain, there is a possibility that anti-CL and/or anti-oxidized phospholipids block the physiological anticoagulant function of CL. For instance, intravenous infusion of monoclonal anticardiolipin antibodies into naïve mice induces the antiphospholipid syndrome. Anticardiolipin antibodies can cause platelet aggregation, and ultimately thrombosis, embolism, and thrombocytopenia. These suggest that CL and anticardiolipin may have opposing effects on platelet aggregation (Schlame, Rua et al. 2000).

Inhibition of CL-dependent protein C pathway by anti-CL might conceivably provide a mechanism for the thrombogenic activity of anti-CL or anti-oxidized CL antibodies (Tsoukatos, Demopoulos et al. 1993; Fernández, Kojima et al. 2000). The general idea is that anti-CL recognizes the interaction between CL and proteins, like β 2-Glycoprotein I. However, Pereira et al presented contrasting results when they isolated two monoclonal antibodies that reacted directly with CL (Pereira, Benedict et al. 1998). However, CL has four acyl chain and is rich in unsaturated fatty acid which makes it highly susceptible to oxidation when coated on the plates. Hence, the epitopes which anticardiolipin antibodies recognize are probably from oxidized species but not from naïve CL per se.

CL and Thyroid dysfunction

Thyroxine is a regulator of mammalian mitochondrial biogenesis. Along with the multiple effects on the composition and function of mitochondria, thyroxine also affects mitochondrial CL content and pathway. An increase in the mitochondrial concentration

of CL upon thyroxin treatment was reported in rat heart and rat liver. These increase ranged from 20 to 50%. In contrast, hypothyroid rats had reduced levels of CL in heart mitochondria (Paradies, Petrosillo et al. 1997; Chicco and Sparagna 2007).

CL with aging, oxidative stress and ischemia disease

Decline of mitochondrial function occurs as one of the early events in aging. An age-related loss of CL was shown in the mitochondria isolated from rat heart, rat liver and in the epidermal cells from humans. Alterations in mitochondrial energy metabolism have been commonly observed in human and animal models of heart failure (HF). Since cardiolipin mainly has polyunsaturated acyl groups, which are susceptible to peroxidative damage, it is conceivable that age-related accumulation of free radicals is responsible for the decline of cardiolipin. Oxidative injury is also relevant to the pathology of ischemia-reperfusion. There is also a report that a small loss of CL observed after ischemia-reperfusion of rat skeletal muscle (Chicco and Sparagna 2007).

CL is rich in mitochondria, but it can be released into the circulation under pathological conditions. Circulating CL have been reported to be extracted from blood samples of hemodialysis patients (Antonopoulou, Demopoulos et al. 1996). CL was also found in the urine of patients with mitochondrial encephalomyopathy. Peitsch and coworkers report that CL can elicit an antibody-independent activation of the complement system (Peitsch, Tschopp et al. 1988). This process probably contributes to the inflammatory response which is typically associated with cardiac ischemia.

Annexin A5 (ANXA5)

Annexins have been described in many organisms from mammals to molds and even plants. Now, more than 13 unique annexin proteins have been found present in vertebrates (Gerke and Moss 2002). Most annexins are abundant intracellular proteins, composing more than 2% of total cellular proteins. Annexins can in a calcium-dependent manner bind to negatively charged phospholipids. They have been implicated in multiple aspects of cell biology. Annexin A1 possesses anti-inflammatory effects, may contribute this function by interacting with PLA2. Recently it was found that Annexin A1 plays multiple roles such as acting as the central mediator of glucocorticoid effect, interfering

with granulocyte extravasation, recruitment, migration, and/or activation at sites of inflammation (Comera and Russo-Marie 1995). Annexin also has antithrombogenic effect, which could be associated with binding to both plasminogen and the tissue plasminogen activator (tPA). Homocysteine can disable the tPA-binding domain of annexin II, thus inhibiting plasmin generation (Hajjar and Krishnan 1999). Antithrombogenic effect can also attribute to Annexin A2 mediating the binding of β 2-glycoprotein I (β 2GPI) to endothelial cells (Ma, Simantov et al. 2000). Conversely, changes in endothelial cell behavior leading to reduced cell surface expression of annexin A2 or metabolic changes that chemically modify annexin A2 can be hypothesized to predispose people to CVD. Annexin II expression within the aortic wall may be associated with the development of an aneurysm (Hayashi, Morishita et al. 2008). Annexins have been shown to inhibit phospholipase A2, protein kinase C, and blood coagulation (Gerke and Moss 2002).

ANXA5 can form two-dimensional crystals on planar lipid bilayers containing negatively charged phospholipids. This binding is likely to affect the properties of membrane such as rigidity, fluidity, and lipid segregation. Hence, this binding may participate in the regulation and/or stabilization of membrane domains. Binding of this annexin to the surface of T cells delays programmed cell death (Brisson, Mosser et al. 1991; Pigault, Follenius-Wund et al. 1994).

Annexin A5 was originally described as an anticoagulant protein, and this activity most likely depends on its Ca^{2+} -regulated binding to anionic phospholipids probably exposed on the surface of activated platelets or endothelial cells (Huber, Berendes et al. 1992; Arispe, Rojas et al. 1996). This binding could thus interfere with the accessibility of such sites for coagulation factors, thereby preventing their local accumulation and activation (Raynal and Pollard 1994; Gerke and Moss 1997).

ANXA5 is a signaling molecule downstream of the gonadotropin releasing hormone (GnRH) receptor in Leydig cells and is involved in steroidogenesis or cell growth in Leydig cells (Brisson, Mosser et al. 1991; Yao and Kawaminami 2008). ANXA5 is also a signaling protein for vascular endothelial growth factor receptor-2(VEGFR-2) by direct interacting with the intracellular domain of the receptor and it appears to be involved in

the regulation of vascular endothelial cell proliferation mediated by VEGFR-2 (Wen, Edelman et al. 1999).

There has been report that ANXA5 imaging is not exclusively valuable for apoptosis detection, but can also be used to visualize inflammation and cell stress (Laufer, Reutelingsperger et al. 2008). Because apoptosis is a potential determinant of plaque instability, annexin V imaging could be a useful noninvasive diagnostic tool, particularly if manipulation of apoptosis evolves as a strategy for plaque stabilization (Kolodgie, Petrov et al. 2003; Laufer, Winkens et al. 2009). Kenis and his coworkers reported that ANXA5 mediated apoptotic cell pinocytic pathway is essential for development and tissue homeostasis of multicellular organisms (Kenis, van Genderen et al. 2004). We have demonstrated that ANXA5 is abundant within advanced human atherosclerotic plaques at the sites of recognized high thrombogenic potential and in SLE decreased ANXA5 binding to endothelium was associated with atherothrombotic cardiovascular disease. These suggest that increasing ANXA5 binding, either by neutralizing aPL, or by administration of ANXA5, could be novel forms of treatment against SLE and CVD (Cederholm, Svenungsson et al. 2005; Anna Cederholm 2007). In this paper we studied the proinflammatory effects of oxCL and the potential antiatherogenic role of ANXA5.

Matrix Metalloproteinases (MMPs)

History of the matrixin family members

In 1962 Gross and Lapiere first reported vertebrate collagenolytic activity in tadpole tissues (tailfin, skin, intestine and gill) undergoing metamorphosis. These tissues are rapidly remodelling the extracellular matrix (ECM) hence triggering apoptosis, cell differentiation and growth.

This discovery stimulated many researchers to look in human tissues, since degradation of collagen is associated with wound healing, tissue regeneration and diseases such as arthritis, cancer, atherosclerosis, aneurysm and tissue ulcerations.

Structure and function of MMPs

MMPs are multidomain zinc metalloproteinases which contain three basic structure distinct domains: an amino terminal propeptide, a catalytic domain, and a hemopexin-like domain. The MMP family is subdivided into five groups based on their primary structure and substrate specificity. They are collagenases, gelatinases, stromelysins, membrane type MMPs and other matrilysin including (MMP7 and MMP26) (Murphy and Nagase 2008).

MMPs, together, are capable of degrading all the constituents of the ECM. They are required for normal development and for general turnover of the ECM. They are tightly regulated at three main points, transcription, activation, and inhibition. All MMPs are secreted as a proform and requires initial activation by another proteinase. Once activated, there are specific MMP inhibitors (tissue inhibitors of metalloproteinases, or TIMPs) which can bind to it irreversibly. MMPs, such as MMP-1 (collagenase 1) are also susceptible to proteolytic cleavage in the hinge region producing N and C terminal fragments which lose there ability to degrade specific substrates, i.e., cleaved MMP-1 can no longer cut triple helical collagen (Nagase and Woessner 1999; Libby 2008).

MMPs and atherosclerosis and CVD

MMPs have been reported to be associated with cancer, arthritis, also report have been implicated in intima thickening, a repair response to damage of the walls of large arteries in human atherosclerotic pathologies, and in the subsequent plaque rupture. Studies using MMP gene knockout mice have indicated that MMP-2 and MMP-9 play key roles in cardiac rupture after myocardial infarction (Romanic, Harrison et al. 2002; Hayashidani, Tsutsui et al. 2003).

The activity of MMPs is essential for atherosclerotic plaque formation. Recruitment of macrophages into the intima of the vessels (if unabated) leads to the secretion of MMPs which can degrade collagen. This may lead to the degradation of the fibrous cap of an atherosclerotic plaque, which can result in acute thrombosis via recruitment and activation of platelets and the coagulation system (Virani, Polsani et al. 2008). Matrix degradation by MMPs may cause plaque instability and rupture that leads to the clinical symptoms of atherosclerosis; unstable angina, myocardial infarction and stroke (George

2000). Close to 60 MMP inhibitors have been pursued as clinical candidates since the first drug discovery program targeting this enzyme family began in the late seventies. Targeted indications included cancer, arthritis, CVD, and many others. However, so far the clinical developments of most MMP inhibitors have been discontinued due to safety reasons and.

Leukotrienes

The proinflammatory lipid mediators known as the leukotrienes (LTs) were initially described according to their bioactivity and were referred to as Slow Reacting Substance of Anaphylaxis (SRS-A) (Brocklehurst 1960). This observation was subsequently explained by LT synthesis induced by phospholipase A2 (PLA2). PLA2 consists of both secretory and cytosolic forms. Cytosolic PLA2 (cPLA2) can liberate glycerol-phospholipids from cell membranes upon activation. Granulocyte-macrophage colony stimulating factor (GM-CSF), PAF, dimethyl sulfoxide (DMSO), transforming growth factor β (TGF β) and 5-lipoxygenase (5-LO) play critical role in the synthesis of leukotrienes (DiPersio, Billing et al. 1988; Werz 2002; Plante, Picard et al. 2006). 5-LO was found primarily in polynuclear leukocytes, macrophages and mast cells. It exists in both the cytosol and the nucleus. cPLA2 displays a calcium-dependent activation, and has a specificity for phospholipids with arachidonic acid (AA) bound at the sn-2 position. It liberates AA from phospholipids, thus providing the substrate for 5-LO. Upon activation, 5-LO is rapidly transported to the nuclear envelope, in close proximity to 5-LO activating protein (FLAP). FLAP is only found in the nuclear membrane and is required for transferal of AA to 5-LO. 5-LO can then catalyze AA into 5(S)-hydroperoxy-6-trans-8,11,14-cis-eicosatetraenoic acid (5-HPETE) and subsequently, 5-HPETE will be transformed into intermediate leukotriene A4 (LTA4). The leukotrienes, consisting of dihydroxy leukotriene LTB4 and the cysteinyl leukotrienes LTC4, LTD4 and LTE4, target cell surface receptors expressed on inflammatory cells and on structural cells of vessel walls. Hydrolysis of LTA4 by LTA4 hydrolase leads to the formation of potent neutrophil chemoattractant LTB4. Conjugation of LTA4 with glutathione through the action of LTC4 synthase yields cysteinyl-leukotrienes LTC4, which then transforms into LTD4 and LTE4 (Figure 5) (Mehrabian and Allayee 2003; Rådmark, Werz et al. 2007).

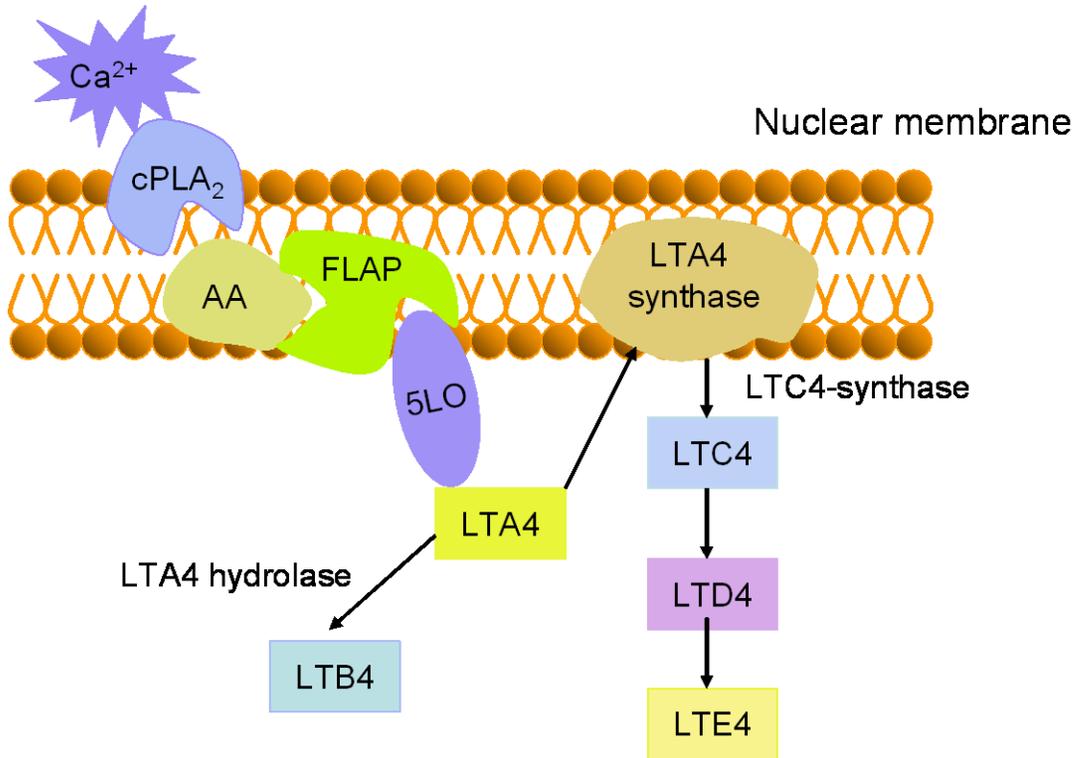


Figure 5. Biosynthesis of leukotriene.

Adapted from Margarete 2003

The production of Leukotrienes can be regulated. T helper cell type 2 cytokines such as IL-4 reduce LTB4 expression in monocytes (Spanbroek, Hildner et al. 2001) however T helper cell type 1 cytokines such as interferon- γ increases 5-LO production. Members of the leukotriene inhibitors have been produced by pharmaceutical companies and are used for the treatment of asthma and inflammatory diseases. However, the results were less than ideal. The reasons are unclear. However, the findings of relationship between cardiovascular disease and leukotrienes have shown great progress in the last 5 years. In 2003, Habenicht and his coworkers showed the evidence that 5-LO can be found in macrophages, dendritic cells, neutrophils and mast cells within the atherosclerotic lesions. In addition, FLAP, LTB4 and cysteinyl-leukotrienes are also identified in the plaques (Spanbroek, Gr bner et al. 2003).

The function of leukotrienes

LTB₄ activate its receptors BLT1 (mainly expressed in the neutrophils), BLT2 (widely expressed) leading to the activation of CD4⁺ and CD8⁺ T cells. Evidences indicate that the leukotriene system is involved in the monocyte adhesion, chemotaxis and transmigration. In addition, LTB₄ is known to be a strong chemoattractant for monocytes as well. Oxidized phospholipids are found to activate LTB₄ receptors and facilitate monocytes recruitment (Harrison and Murphy 1995). Antagonist of LTB₄ can decrease monocyte chemoattractant protein-1 (MCP-1) production. BLTR antagonist inhibits integrin expression on monocytes. 5-LO and LTB₄ have been found to induce IL-1 β production which plays a direct role in hyperplasia and SMC proliferation. In the presence of 5-LO or FLAP inhibitors, production was impaired and VCAM-1 expression induced by IL-1 β was reduced (Devaraj and Jialal 1999). LTB₄ is also found to be an activator of peroxisome proliferator-activated receptor (PPAR)- α which can potentiate inflammation in the lesions.

Interestingly, 5-LO is abundantly expressed in the atherosclerotic lesions of apolipoprotein E^{-/-} and LDL^{-/-} mice. Recent studies have shown that the 5-LO pathway is implicated in human atherosclerotic plaque stability and in murine aortic aneurysm development. Hence, in line with this notion blockade of this pathway could prevent plaque rupture and pathological sequelae of arterial remodeling (Zhao, Moos et al. 2004; Cipollone, Mezzetti et al. 2005). It was also shown that with the administration of LTB₄ receptor antagonist, foam cell formation was reduced in these mice (Aiello, Bourassa et al. 2002). ECM metalloproteinase (MMPs) inducers can activate phospholipase A₂ to release AA, subsequently processed by 5-LO, to generate 5-HETE, leading to MMP-1 and 2 productions.

Leukotriene pathway is involved in the regulation of vascular tone. CysLT₁ has been shown to modulate angiotension II, thus stimulating ECM production and SMC growth. FLAP inhibitors have been shown to decrease angiotension II production (Zukowska-Grojec, Bayorh et al. 1985; Ishizaka, Nakao et al. 1999)

Anti-PC and other novel risk markers in CVD

CRP and CVD

The possibility of using c reactive protein (CRP) to predict the risk of first vascular event was first shown in the Physicians' Health study. A total of 22,000 healthy US male physicians were enrolled and it was shown that men with baseline CRP values in the highest quartile had three times the risk for myocardial infarction (MI; RR of 2.9) and two times the risk for stroke (RR of 1.9) as compared to men in the lowest quartile of CRP values, independent of traditional risk factors (Ridker, Cushman et al. 1997). Studies have shown that CRP can predict risk of first events in women as well (Ridker, Buring et al. 1998). However, Danesh et al, in a case-control study of 2459 cases and 3969 controls from the prospective Reykjavik study, showed that the predictive ability of CRP was only modest (Danesh, Wheeler et al. 2004). Together with evidence from other studies, the usage of CRP values as a clinical tool remains debatable (Miller, Zhan et al. 2005; Khera, de Lemos et al. 2006).

Lp-PLA2

Lp-PLA2 is primarily secreted by macrophages with approximately 70% to 80% bound to LDL. Lp-PLA2 is a proinflammatory enzyme associated with increased risk of CHD and stroke. Lp-PLA2 leads to the formation of two molecules: lysophosphatidylcholine and oxidized nonesterified fatty acids, both of which have been shown to upregulate inflammation and lead to the influx of more inflammatory cells into the intima of blood vessels. There is also evidence to suggest that Lp-PLA2 localizes in the thin fibrous-cap of plaques, Hence causing these plaques to be more prone to acute rupture (Kolodgie, Burke et al. 2006). Health Study suggested that although the baseline Lp-PLA2 levels were significantly higher in cases as compared to controls, the levels were not predictive of future CHD or stroke after adjusting for other cardiovascular risk factors. It is thus debatable whether Lp-PLA2 has clinical utility (Virani, Polsani et al. 2008).

Anti- PC

Phosphorylcholine (PC) was first detected in 1967 in the Gram-positive bacterium *Streptococcus pneumoniae*, It was found to be associated with a polysaccharide component of the cell wall, subsequently shown to be teichoic acid. Later, it was discovered to be a structural component of a wide variety of prokaryotic and eukaryotic pathogens. In some cases, PC in infectious agents can benefit the infected host due to its targeting by both the innate and adaptive immune responses (Harnett and Harnett 1999).

Immunization with PC in animal models, such as mice, has shown evidence of a significant increase in the number of splenic mature B cells. Expression of major histocompatibility complex class II antigens was found decreased and B-cell clusters were detected in plaques. In addition, serum from PC-KLH vaccinated mice was able to reduce macrophage-derived foam cell formation in the presence of oxLDL in vitro. Immunization with oxLDL reduces experimental atherosclerosis independently of CD4⁺ T-cell help. The main epitope of anti-oxLDL antibodies is not contained in the protein portion of the molecule but rather in the phospholipids and has been identified as phosphorylcholine (PC) (Caligiuri, Khallou-Laschet et al. 2007). Binder et al found that many autoantibodies to oxLDL from mice share complete genetic and structure identity with antibodies from the classic anti-PC B cell clone, T15 and vaccination with pneumococcae rich in PC in the cell membrane could increase the levels of oxLDL IgM and lead to the expansion of T15 IgM-secreting B cells primarily in the spleen. The size of atherosclerotic lesion formation from LDL^{-/-} mice was also reduced (Binder, Horkko et al. 2003). Natural antibodies have been shown to be produced by B1 cells in mice in an IL-5 dependent manner (S. Bao 1998; Erickson, Foy et al. 2001). Natural antibodies EO6 have been suggested to play an important role in inhibiting PC-containing oxPL induced IL-6 expression, suggesting anti-inflammatory effects of these natural antibodies (Imai, Kuba et al. 2008).

From the European Lacidipine Study, we followed 240 individuals with established hypertension for four years and found that IgM anti-PC were negatively associated with atherosclerosis development. It is also noteworthy that high levels of anti-PC were associated with a favourable outcome in hypertensive patients (Su, Georgiades et al. 2006). Follow up study have suggested that levels of antibodies were stable in patients. In

Malmö Diet Cancer study, 4232 subjects (2039 men and 2193 women, age older than 60years old) were included. They were followed from 5 to 7 years. We found that low levels of PC-specific IgM antibodies were associated with an increased risk of ischemic stroke in men (Sjöberg, Su et al.). Low IgM anti-PC could probably be a novel risk marker among men for ischemic stroke and cardiovascular disease (Frostegard, Tao et al. 2007). Further studies are required for women.

Autoimmunity

Failure to distinguish self from nonself by the host's humoral and cellular immune systems, results in attack on self cells and organs by autoantibodies and self-reactive lymphocytes. A number of mechanisms exist to protect an individual from potential self-reactive lymphocytes. A state of unresponsiveness to an antigen is defined as tolerance. Central tolerance, occurs in the primary lymphoid organs, namely, the bone marrow, and thymus which eliminates T-B-cell clones before the cells are allowed to mature if they process receptors that recognize self antigens with greater than a low threshold affinity. Peripheral tolerance renders lymphocytes in secondary lymphoid tissues inactive or anergic. Regulation through apoptosis is another important mechanism to maintain the tolerance. Despite this system of regulation, self-reactive clones of T and B cells are occasionally activated, generating humoral or cell mediated responses against self antigens. These inappropriate responses from the immune system against self components are called autoimmunity. Autoimmune reactions can cause serious damage to cells and organs, sometimes with fatal consequences (CarneiroSampaio, Coutinho et al. 2007; Lleo, Selmi et al. 2008).

Systemic lupus erythematosus (SLE)

“Lupus erythemateux” was first described by Cazenave and Clausit in 1852. It is a prototypic autoimmune disease characterized by autoreactive T cells and polyclonally activated B cells that produce autoantibodies directed against self antigens, defective clearance of apoptotic cells and immune complexes; and are associated with diverse clinical manifestations (Cervera 2006). The involvement of various organ systems often

leads to secondary morbidities resulting from renal failure, hypertension, and atherosclerosis (Mok and Lau 2003).

The life expectancy of SLE patients has improved from an approximate 4-year survival rate of 50% in the 1950s to a 15-year survival rate of 80% today. Even so, more than 15% young patients die mostly from lupus or infection. Later, myocardial infarction and stroke became important causes of death (Rahman and Isenberg 2008). SLE is thought to result from complex interactions among environmental, hormonal, and genetic factors.

Pathogenesis

Sex hormone

Since 90% of patients with lupus are female, It is likely that female hormones play an important role. However, it is unclear on how sex hormones could promote lupus. Estrogen has been proposed to modulate pathogenic naïve B cell activation(Grimaldi, Jeganathan et al. 2006), increase calcineurin expression which leads to dephosphorylation of nuclear factor of activated T cells(Rider, Foster et al. 1998), increase CD40-L expression to activate T cells (Rider, Jones et al. 2001) and reduce T cell apoptosis (Evans, MacLaughlin et al. 1997).

It is also reported that estrogen can activate CD22, SHP-1, and Bcl-2 in B cells (Grimaldi, Cleary et al. 2002) However, trials of hormonal treatments for lupus, such as dehydroepiandrosterone, have been disappointing. HLA DR2 and DR3 gene polymorphisms have been found to be associated with the susceptibility to SLE (Graham, Ortmann et al. 2007).

Autoantibodies

The central immunological disorder in SLE is the production of autoantibodies. These autoantibodies are mostly against nuclear materials. Anti-double strand DNA and anti-Sm antibodies are unique to the patients. Levels of anti-sm antibodies are usually constant in the patients and anti-DNA antibodies show preferential deposition as DNA-anti-DNA immunocomplex in the kidneys which suggests that they are associated with glomerulonephritis (Foster, Cizman et al. 1993).

Cytokines

IL-10 is more common in SLE patients than in controls, resulting in autoantibody production by B cells (A. Csiszár 2000). However, anti-PC antibodies are believed to be produced by B1 cells in mice, an observation reported by several groups. Our group has reported that anti-PC have potential protective roles of in the development of atherosclerosis in SLE and SLE-related CVD. In mice, IL-5 is necessary for B1 cells to produce anti-PC antibodies. PC can stimulate CD5-positive B1 cells to produce IL-10. The production of nature antibodies can occur in the complete absence of external antigenic stimulation, but can be enhanced later in life by positive selection. The role of nature antibodies in SLE and the prospect that high levels of IL-10 in SLE may inhibit anti-PC antibodies production remain to be elucidated.

Defective clearance of immunocomplex

Defective clearance of apoptotic cells was considered to be one of the disease mechanisms for SLE. The reasons for defective clearance of apoptotic cells in SLE are not fully understood. Complement plays an important role in the host innate immune defense and the clearance of immunocomplex and apoptotic cells in the body. The role of complement is somewhat paradoxical in SLE. However, reports have shown that defects of early complement components, such as C1q, C2 and C4, could contribute to the failure of autoantigen clearance in the body. Patients or mice with C1q deficiency develop autoantibodies and lupus-like syndrome (Botto, Dell'Agnola et al. 1998). Over production of late stage product such as e terminal product C5b-9 can cause inflammation and host cell damage. Our data from 78 SLE cohort provided evidence of lower serum levels of serum C3 and C4 in patients as compared to controls and serum from SLE patients can stimulate macrophage to produce more complement C5b-9 in vitro (unpublished). The role of natural anti-PC in the development of SLE is still not clear. We already know that PC is widely expressed on the apoptotic cell membranes. Probably anti-PC antibodies might play a role in apoptotic cells, Therefore a deficiency in anti-PC antibodies might contribute to the disease development. We found that the levels of anti-PC were decreased in SLE and SLE-related CVD as compared to controls. However, this cohort has been designed to study CVD in SLE and not for SLE per se. The role of anti-PC in SLE remains to be further studied. Studies of B1 cells in SLE and if they have an impaired production of natural antibodies might answer some of these questions.

Clinical symptoms

The clinical course of SLE is variable and may be characterized by periods of remissions and of chronic or acute nature. The most common pattern is a mixture of constitutional complaints with skin, musculoskeletal, hematologic, renal, or central nervous system manifestations (Cervera 2006) (Table 1).

Table 1 Manifestation of systemic lupus erythematosus

Systemic	Fatigue, fever, weight loss
Musculoskeletal	Arthralgias, myalgias, non-erosive polyarthritis
Cutaneous	Malar rash, photosensitivity, ulcerations
Neurologic	Cognitive disturbances, psychoses, seizures
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

Diagnosis

According to the updated 1982 ACR criteria, the presence of one or more of the four elements: 1) hemolytic anemia (with reticulocytosis); 2) leukopenia (<4000/microL on two or more occasions); 3) lymphopenia (< 1500/microL on two or more occasions); or 4) thrombocytopenia (< 100,000/microL in the absence of offending drugs) is now considered as a single hematologic disorder (Table 2). The sensitivity and specificity of the individual elements of the hematological criteria range from 18% to 46% and 89% to 99%, respectively (Gilboe and Husby 1999; Kao, Manzi et al. 2004).

Table 2 Diagnosis (four of this criteriae are fulfilled)

The 1997 American College of Rheumatology criteria for classification of SLE	
1. Malar rash	Fixed erythema, flat or raised, over the malar eminences
2. Discoid rash	Erythematous raised patches with adherent keratotic scaling and follicular plugging
3. Photosensitivity	
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 documented by ECG, 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
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

The SLICC=ACR damage index for SLE was developed to assess accumulated damage since the onset of the disease. It is recommended as an outcome measure for longitudinal studies of prognosis and response to new therapies, and as a stratification measure for clinical trials (Gladman and Urowitz 1999) .

SLE and CVD

The risk of CVD in SLE is extremely high, women aged 44–50 had a 50 times increased risk of myocardial infarction (MI) as compared with controls, according to the Framingham study and the relative risk for coronary heart disease was 7.5, after adjusting for Framingham risk factor (Svenungsson, Jensen-Urstad et al. 2001; Cederholm, Svenungsson et al. 2005; Frostegard 2008). Analysis of the California Hospital Discharge Database between 1991 and 1994 has shown that young women (18 to 44 years of age)

with lupus were 2.27 times more likely to be hospitalized for acute MI. Similarly, studies of the Pittsburgh Lupus Cohort revealed a 52-fold increase in cardiovascular events in 35- to 44-year old women with SLE., Cardiovascular events remain increased in SLE even after controlling for Framingham Heart Study risk factors (Salmon and Roman 2008). There is increasing evidence that chronic inflammation contributes to accelerated atherogenesis and plays a role in all stages of atherosclerosis (i.e., atherogenesis, atheroma progression, and the development of thrombosis) (Frostegård, Ulfgren et al. 1999). The increased cardiovascular morbidity and mortality in patients with RA and SLE cannot be entirely explained by traditional risk factors (Svenungsson, Jensen-Urstad et al. 2001; Anna Cederholm 2004; Frostegard 2005; Johan Frostegård 2005).

Treatment of SLE

General measures may be taken by the patient to help limit the onset and severity of a flare. Probably the most important of which for many patients, particularly those with skin involvement, is the avoidance of over-exposure to sunlight (Ioannou and Isenberg 2002). The pharmacological management of SLE patients presently revolves around four main classes of drugs, often in combination: non-steroidal anti-inflammatory drugs (NSAIDs), antimalarials, corticosteroids and cytotoxic drugs. Rituximab (RTX) has been widely used in clinics in the recent years. CD20 regulates the early step in the activation process and is needed for cell cycle initiation and cell differentiation. RTX is a chimeric monoclonal IgG1 antibody which binds selectively to CD20 antigen with high affinity. The antibody Fab domain (murine) of RTX binds to the CD20 antigen on B cells while the Fc domain (human) recruits immune effect cells which functions to mediate B cell lysis (García-Carrasco, Jiménez-Hernández et al.). Anolik has shown evidence from SLE patients that specific B cell depletion therapy with rituximab dramatically improves abnormalities in B cell homeostasis (Jennifer H. Anolik 2004). Other reports provided evidence that Rituximab without concomitant immunosuppressive therapy could improve disease activity scores in mild to moderate SLE patients (Albert, Dunham et al. 2008); and B cell depletion not only offers the prospect of sustained disease remission (K. G. C. Smith 2006; Sabahi and Anolik 2006), but may also stop the progression of lupus nephritis (Ding, Foote et al. 2008). However, Rituximab was also reported to cause

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infectious complications. There is also a possibility that B cell depleting therapy may decrease B1 cell population which is important for natural antibodies production. These antibodies may have atheroprotective functions.

Aim of the study

- The aim of this study is to investigate:
- by what mechanisms anti-PC may protect against CVD
- the role of inflammatory phospholipids like LPC and others including CL in atherosclerosis and CVD
- if ANXA5 and anti-PC has any potential therapeutic role in CVD by interfering with inflammatory phospholipids
- to study the role of dyslipidemia and anti-PC in SLE

Patients and methods:

Study group (paper I, IV)

The study group consisted of 26 women with SLE who had survived one or more manifestations of CVD (myocardial infarction, angina pectoris, thromboembolic stroke or intermittent claudication), 26 age-matched women with SLE and no clinical manifestations of CVD and 26 age-matched control women who were recruited randomly using the Swedish population registry. Details of the recruitment and clinical characteristics of the three groups have been reported (Svenungsson, Jensen-Urstad et al. 2001). All patients fulfilled the 1982 revised criteria of the American Rheumatism Association for classification of SLE. The study was approved by the local Ethics Committee of the Karolinska Hospital. All subjects gave informed consent before entering the study.

Carotid Ultrasound (paper I, IV)

The right and left carotid arteries were examined with a duplex scanner (Acuson Sequoia, Mountain View, California, USA) and the intima-media thickness (IMT) was determined as described (Lemne, Jogstrand et al. 1995). A plaque was defined as a local intima-media thickening, with a thickness greater than 1 mm.

Lipoprotein particle size and affinity of LDL to proteoglycans (paper IV)

The characteristics of plasma lipoprotein particles were determined by NMR techniques as described originally by Otvos and coworkers (Cromwell and Otvos 2004); the measurements were performed by LipoScience, Inc. Ca, USA.

Plasma LDL-binding to glycosaminoglycans (GAGs) was performed by EMSA as described (Linden, Bondjers et al. 1989; Hurt-Camejo, Paredes et al. 2001). In short, the movement of GAGs through a non-denaturing agarose gel is hindered when LDL particles are bound to it. After electrophoresis of solutions containing LDL; or LDL and GAGs, bands that correspond to a migration of complexes, as well as of free GAGs, can be detected by staining with toluidine blue.

Cell culture

Macrophage differentiation from mononuclear cells of the Buffy coat (paper II)

Human mononuclear cells were isolated from freshly prepared buffy coats (Karolinska Hospital Stockholm, Sweden) by gradient centrifugation on Ficoll-Paque (Amersham Biosciences, Uppsala, Sweden). The mononuclear cells were cultured at a density of 7×10^6 streptomycin and 10% FBS 10^6 /ml in RPMI-1640 medium with 25mM Hepes, 1% L-glutamine and 1% penicillin. After 7 days, there are approximately 1×10^6 macrophages per well.

Macrophages differentiated from THP-1 cells (paper II, III)

Human monocyte-derived THP-1 cells (American Type Culture Collection, Manassas, VA, U.S.A.) were grown in RPMI-1640 medium, 10% FBS supplemented with 1mM sodium pyruvate, 0.05mM β -mercaptoethanol, penicillin (100U/ml), and streptomycin (100 μ g/ml) in a humidified cell incubator with 5% CO₂ at 37 °C. THP-1 monocytes were differentiated by incubation with 0.1 μ g/ml PMA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) for 1-5 days.

Neutrophils (paper II)

Human polymorphonuclear cells (PMNs) were isolated from freshly prepared buffy coats (Karolinska Hospital blood bank, Stockholm, Sweden) by dextran sedimentation, hypotonic lysis of erythrocytes and gradient centrifugation on Lymphoprep (Axis-Shield PoC AS, Oslo, Norway). PMNs were suspended at a density of 10×10^6 /ml in Dulbecco's PBS (Gibco (Invitrogen), Paisley, UK). PMN purity (> 95%) and viability (> 98%) were determined using Hemacolor (J.T. Baker, Utrecht, Holland) and Trypan Blue (Sigma Chemical Co.) staining, respectively.

Pooled human umbilical vein endothelial cells (paper I, II, III)

Pooled human umbilical vein endothelial cells (HUVECs) at passage 2 were purchased from Cascade Biologics, Inc (Portland, OR). Cultures were maintained in EGMTM phenol red-free medium (Clonetics, San Diego, CA), containing 2% of FBS and

supplements, at 37°C under humidified 5% CO₂ conditions. All experiments were performed at passages 3 to 5. HUVECs were seeded at 6x10⁴ cells/2mL density on 6-well plates (NUNC Inc, Naperville, Ill). The endothelial cells were left overnight for attachment to occur before they were ready for stimulation.

Co-culture of HUVECs and macrophages (paper III)

HUVECs (1x10⁵ cells) were seeded in 6-well culture plates (Nunc, Naperville, IL, USA) containing in 1 ml complete EBM and cultured for 24 h at 37°C. The cell layer was subsequently washed twice with EBM and cultured either alone or in co-culture with macrophages (1x10⁵ cells) in 0.5 ml complete EBM. In parallel, macrophages (1x10⁵ cells) were cultured alone in 0.5ml complete EBM. In some experiments, LPC or oxLDL was added to the co-cultures at the start of the culture. In addition, some co-culture experiments were pre-incubated with ANXA5 for 1h before oxLDL or LPC was added to the cultures. After 24h of incubation the cells were detached via trypsinisation and collected by centrifugation (180g for 7 min). The HUVECs and the macrophages were separated from each other by trypsinisation and thereafter collected by centrifugation. The viability of HUVECs and macrophages, whether cultured alone or in co-culture, was more than 80% as assessed by propidium iodide (final concentration 1.0 mg/ml) (Sigma, St. Louis, MO, USA).

Enzyme-linked immunosorbent assay (ELISA)

ANXA5 Binding to Antigen (paper II)

F96 microtiter polysorp plates (Roskilde Denmark) were coated with oxCL, CL or Hydro Heart CL (Biosearch Technologies, Inc, Ca, USA) at 5µg/ml and then incubated overnight at 4⁰C. After five washings with PBS, the plates were blocked with 2% PBS-BSA for 2h at room temperature. ANXA5 were added and the plates were incubated for 1h. After washing, bound ANXA5 was detected by subsequently incubating with rabbit anti-human annexin V polyclonal antibodies (Hyphen Biomed, Andresy, France) 1:2000 and polyclonal goat anti-rabbit Immunoglobulins/AP (DakoCytomation) 1:3000. The reaction was developed with alkaline phosphatase as substrate (Sigma), and optical density (OD) was read at 405 nm with an ELISA Multiscan Plus spectrophotometer

(Molecular Devices Emax, San Francisco). All samples were measured in duplicates and the coefficient of variation was below 15%.

Determination of autoantibodies against PC-BSA, PC-KLH, PS and BSA (paper II)

IgG and IgM antibodies to PC-BSA, PC-KLH, PS and BSA were determined by ELISA. F96 microtiter polysorp plates (Roskilde, Denmark) were coated with PC-BSA or PC-KLH (Biosearch Technologies, Inc., CA, USA) at 10 μ g/ml, BSA (Sigma-Aldrich Chemie GmbH, Steinheim, Germany) at 2 μ g/ml; While Immulon 1B plates (Thermo Labsystems, Franklin, MA, USA) were coated with PS (Sigma) 160 μ g/ml, incubated overnight at 4°C. According to the manufacturer's description, each KLH molecule contained eight PC while each BSA molecule contained two PC. After five washings with PBS, the plates were blocked with 2% PBS-BSA for 2 h at room temperature. Serum samples were diluted 1:30 in 0.2% BSA-PBS, added to the coated plates at 50 μ l/well and the plates were then washed as described earlier. Subsequently, the plates were incubated with 100 μ l/well of alkaline phosphatase-conjugated goat anti-human IgG (Sigma) at 1: 9000 dilution or alkaline phosphatase-conjugated goat anti-human IgM (Sigma) at 1: 7000 dilution, The reaction was developed with alkaline phosphatase substrate (Sigma) and optical density (OD) was read at 405 nm with an ELISA Multiscan Plus spectrophotometer (Molecular Devices Emax, San Francisco, CA, USA). All samples were measured in duplicates and the coefficient of variation was less than 15%.

IgG anti-PC characterization (paper I)

The binding specificity of the purified IgG anti-PC was measured by competition ELISA. The purified IgG anti-PC was pre-incubated with indicated concentrations of competitors, including PC-KLH, phosphatidylcholine (PTC) and KLH. The supernatants were added to microtiter plates (Nunc, Roskilde, Denmark) coated with PC-KLH (10 μ g/ml) and the amount of bound antibody was detected with alkaline phosphatase-conjugated goat anti-human IgG. The colour was developed by adding the alkaline phosphatase substrate. While the percentage of inhibition was calculated with $(OD \text{ without competitor} - OD \text{ with competitor}) \times 100\% / OD \text{ without competitor}$.

CETP activity (paper IV)

Commercially available kits were used for the measurement of serum/plasma CETP activity by ELISA according to the manufacturer's instruction (Biovision, Ca, USA).

Analysis of LTB₄ biosynthesis by Enzyme Immunoassay (EIA) (paper II)

1×10^6 macrophages differentiated from human mononuclear cells were incubated with different agents according to the experimental design, and then quenched with an equal volume of methanol. After being acidified to pH 3-4, the samples were purified by solid-phase extraction (SupelcleanTM LC-18, Supelco) and eluted in methanol. After evaporation of the solvent under a stream of nitrogen, the samples were resuspended in EIA buffer. The level of LTB₄ was determined with LTB₄ EIA kit (Cayman Chemical) by using dilutions within the linear portion of the standard curve.

Flow cytometry analysis

MMP-9 measurement (paper III)

The cell suspensions, including HUVECs, macrophages, and co-cultured HUVECS and macrophages, were washed twice with PBS. The cells were fixed in 2% paraformaldehyde for 15 min at room temperature (RT) and washed with PBS prior to permeabilization with PBS containing 0.1% Saponin (SAP buffer) (15 min, RT). After washing with SAP buffer, the cells were stained for surface and intracellular MMP-9 in the dark for 30 min at RT using fluorescein isothiocyanate (FITC)-labeled mAb (56129). The mAb was titrated in preliminary experiments and a negative control with an isotype-matched antibody was included (clone). After two washing steps with SAP buffer, the cells were resuspended in 300µl PBS and analyzed in a FACSCaliburTM flow cytometer using CellQuest software (Becton & Dickinson, San Jose, CA, USA). Between 10 000 and 30 000 events per test were acquired. To analyze the expression of MMP-9, a gated area was selectively determined for the cells using forward vs. side scatter parameters in order to discriminate between the fibroblasts and monocytes. The results obtained were expressed as the frequency of positive cells and the mean fluorescence intensity (MFI) of MMP-9 referred to staining with the isotype control mAb (clone).

oxLDL uptake measurement (paper II, III)

Labelling oxLDL by Dil

oxLDL (Industrylane Frederick) was incubated with Dil (Molecular Probes Engine, Oregon, USA) in lipoprotein-deficient serum (Sigma) at 37 °C for 15h. Thereafter, oxLDL was dialyzed against saline-EDTA buffer for 6 hours.

oxLDL uptake inhibited by oxCL

The macrophages differentiated from THP-1 cells were seeded in a 24-well plate (NUNC Inc, Naperville, Ill) at a density of 3×10^5 cells/well in DMEM (INVITROGEN, USA) containing 10% FBS overnight. The cells were then incubated with Dil-oxLDL (5µg/ml), oxCL (7, 10µg/ml) and CL control (7, 10µg/ml) for 4h. Thereafter, the cells were washed 4 times with 0.2% BSA/PBS and once with PBS. The cells were subsequently harvested in PBS containing 0.1% BSA and 0.01% NaN₃. Uptake of Dil-labelled oxLDL was studied by flow cytometry and a minimum of 10,000 cells were analyzed.

oxLDL uptake inhibited by ANXA5

The macrophages were seeded in a 24-well plate at a density of 2×10^5 cells/well in DMEM (INVITROGEN, USA) containing 10% FBS overnight. The cells were then incubated with Dil-oxLDL (5µg/ml), oxCL (40, 80µg/ml), CL control (40, 80 µg/ml), unlabeled oxLDL (40µg/ml) or unlabeled LDL (40µg/ml) for 4h. For the experiment of ANXA5 inhibition of Dil-oxLDL, macrophages were incubated with Dil-oxLDL (5ug/ml), together with various concentrations of ANXA5 (0.01, 0.04, 0.16, 0.64, 1, 10, 20, 40µg/ml). Thereafter, the cells were washed 4 times with 0.2% BSA/PBS and once with PBS. The cells were then harvested in PBS containing 0.1% BSA and 0.01% NaN₃. Mean fluorescence intensity was measured by flow cytometry (BD Biosciences, San Jose, CA, USA). For each sample, fluorescence emission above 550 nm was measured and a minimum of 10,000 cells were analyzed.

Measurement of adhesion molecules expression upon oxCL stimulation (Paper II)

oxCL and CL (10µg/ml) were added into HUVEC cells from each well. For the ANXA5 (Bender MedSystems GmbH, Austria) inhibition study, oxCL was incubated with

ANXA5 (20µg/ml) for 30mins before being introduced to the cells. After 24h incubation, detached floating cells were washed away and the remaining cells were harvested into Falcon FACS tubes. After centrifuging at 1200 rpm for 5mins, the cells were resuspended in 300µl FACS buffer (1% FBS-PBS) and incubated with 10µl PE-conjugated anti-CD54 (eBioscience) and 10µl FITC-conjugated anti-human CD106 (Becton, Dickinson) for 30 mins on ice. The intercellular adhesion molecule (ICAM-1) CD54 and vascular cell adhesion molecule (VCAM-1) CD106 were studied with flow cytometry analysis equipped with CellQuest software (BD Biosciences, San Jose, CA, USA). For each sample, 10,000 events were analyzed.

Inhibition of inflammation by anti-PC (paper I)

HUVEC cells were incubated with PAF (1µg/ml) and either of anti-PC IgG(10µg/ml) or commercially available human Ig Gammagard®S/D (Baxter, Inc.) (10µg/ml). After 24 h of incubation, detached floating cells were washed away and the remaining cells were harvested into BD Falcon tubes (Becton Dickinson, San Jose, CA, USA). After centrifuging at 410g for 5mins, cells were resuspended in 300 µl FACS buffer (1% FBS–PBS) and stained with PE-conjugated anti-CD54 and FITC-conjugated anti-human CD106 for ICAM and VCAM analysis.

Chemical treatments of CL (paper II)

CL from bovine heart was purchased, as dissolved in ethanol solution from Sigma (Sigma product C1649) and stored at –20°C. Hydro heart CL (reduced CL) was purchased from Avanti Polar Lipids, Inc. To generate saturated molecular species, CL was oxidized in aqueous solutions containing 1.5mmol/L tert-butylhydroperoxide and CuSO₄ in concentrations ranging from 20µmol/L. Both native CL and copper-treated CL were analyzed by mass spectrometry to confirm that CL had been oxidized by copper and tert-butylhydroperoxide.

Intracellular calcium mobilization (paper II)

Neutrophils were added into black, 96-well plates with transparent bottom (Corning Costar; 5×10^4 cells/well), and the plates were spinned down at 120g for 3mins, afterwards changing the medium containing 4 µM FURA-2AM (Fura-2 acetoxymethyl

ester), or buffer as appropriate, and the cells were incubated for 30 min at 37°C and 5% CO₂. The cells were washed four times with 50 µl of a buffer solution (135 mM NaCl, 4.6 mM KCl, 1.2 mM MgCl₂, 1.5 mM CaCl₂, 11 mM glucose, 11 mM Hepes, pH 7.4) before a final 45 µl of buffer was added to each well.

The plates were transferred to a fluorometer (Fluostar™, BMG Technologies), and 50 µl of different agonists according to experimental design or buffer solution as control were injected into individual wells, The cells were then monitored for the next 120s. Control wells containing cells that had not been exposed to FURA-2AM were used to subtract for background auto-fluorescence. The results were given as the ratio of mean fluorescence intensity (MFI) between 340 and 380 nm, and normalized by control.

Extraction of IgG anti-PC from human Ig (Paper I)

Column coupling

PC-KLH (Biosearch Technologies Inc., CA, USA) was diluted in coupling buffer (0.2 M NaHCO₃, 0.5 M NaCl, pH 8.3) to 1 mg/ml and then coupled to a HiTrap NHS column (Amersham Biosciences, Piscataway, NJ, USA) according to the manufacturer instruction. The coupled column was stored at 4°C.

Purification of IgG anti-PC

Human pooled immunoglobulin (Baxter Medical AB Torshamnsgatan 35 Stockholm, Sweden) was diluted in binding buffer (20 mM Na₂HPO₄) to 50 mg/ml and filtered through a 0.45 µm filter before passing through a pre-coupled PC-KLH sepharose gel column. Washing and further elution were performed according to the manufacturer's recommendation. Shortly, anti-PC IgG was eluted by 0.1 M glycine-HCl buffer, and the pH value was neutralized to 7.0 by 1 M Tris-HCl (pH 9.0). The purified fractions were desalted using PD-10 columns (Amersham Pharmacia Biotech AB, Warrenale, PA, USA). The antibody was concentrated by centrifugation with Centriprep Centrifugal Filter (Millipore, USA, Billerica, MA, USA), then stored at 4°C after being filtered through a 0.22 µm filter.

Statistical analysis

The statistics were computed using StatView software (SAS Institute AB, Göteborg, Sweden). Antibody levels were dichotomized at the 75th and 90th percentile or determined as continuous variables as indicated. Correlation analysis was performed using simple regression for normally distributed variables, and Spearman's correlation analysis for non-normally distributed variables; Skewed continuous variables were logarithmically transformed to attain a normal distribution. Study groups were compared using ANOVA for continuous variables with Fischer's protected least significant difference test as post hoc test and chi-squared for categorical variables where comparisons between two groups were made using Fischer's exact test. The significance level was determined at $P < 0.05$.

Results and Discussion

IgG anti-PC levels in SLE and SLE-related CVD

In **Paper I**, the study group consisted of 26 women with SLE with a history of CVD (myocardial infarction, angina pectoris, thromboembolic stroke or intermittent claudication), 26 age-matched women with SLE but without clinical manifestations of CVD and 26 age-matched control women. IgG anti-PC-BSA and anti-PC-KLH were decreased among SLE-cases and SLE-controls as compared to controls, respectively. SLE cases were more prevalent in the lowest 25th percentile of IgM (and IgG) anti-PC as compared to controls. Among SLE controls, anti-PC-BSA were negatively associated with organ damage (SLICC) and disease activity (SLEDAI). Among SLE cases, anti-PC-BSA and anti-PC-KLH were negatively associated with SLICC and anti-PC-BSA was negatively associated with SLEDAI.

Anti-inflammatory effect from IgG anti-PC

In **Paper I**, IgG anti-PC were subtracted from the human Ig and the specificity of IgG anti-PC was confirmed by competition ELISA. PAF-induced expression of adhesion molecules by HUVECs could be inhibited by IgG anti-PC suggesting an atheroprotective role of anti-PC.

Novel inflammatory effect of oxCL

In **Paper II**, CL was oxidized in vitro and this oxidation was confirmed by mass spectrophotometer. oxCL can stimulate IL-6 production and significantly induce HUVECs to express ICAM-1 and VCAM-1. It can also activate cells and provoke intracellular calcium mobilization. The production of LTB₄ from macrophages was significantly induced when cells were exposed to oxCL. CL, however, failed to show these effects.

ANXA5 inhibit oxCL induced cell activation and inflammation

In **Paper II**, cell activation and inflammatory effects induced by oxCL could be inhibited by ANXA5. ANXA5 was found to bind to oxCL. In conclusion, oxCL but not native CL

can cause inflammatory effects. These effects caused by oxCL could contribute to human chronic inflammatory disease in general and CVD. ANXA5 could have potential therapeutic roles in anti-inflammation.

Pro-inflammatory effects of oxLDL and LPC

In **Paper III**, oxLDL and LPC could stimulate the expression of MMP-9 in co-cultures of HUVECs and macrophages, LPC could also induce macrophage production of LTB₄, LDL or PTC had no effect on these parameters.

ANXA5 inhibit MMP-9 expression and oxLDL uptake

In **Paper III**, the expression of MMP-9 induced by oxLDL and LPC could be inhibited by ANXA5. Preincubation of ANXA5 was able to in a concentration dependent manner inhibit macrophages uptake of oxLDL. The inhibition of oxLDL uptake was up to 70%. These results suggest that oxLDL and LPC are probably involved in the plaque inflammation process and plaque rupture, ANXA5 could play a role in the anti-inflammation and inhibition of foam cell formation thus preserving plaque stability properties.

Lipoprotein pattern in SLE and SLE related CVD

In **Paper IV**, sdLDL were not raised in SLE and SLE related CVD. The lipoprotein pattern in SLE and SLE related CVD is not typically proatherogenic but that autoantibodies against apolipoprotein A1 and lipoprotein lipase (aApo A-1 and aLPL) were associated with CVD in SLE (data not shown). Small HDL, often assumed to be less atheroprotective than larger HDL-particles, were also found to be less common in both SLE cases and SLE controls. The lipid profile in SLE-related CVD does not appear to be highly atherogenic.

Conclusions:

Anti-PC can be extracted from human Ig and has the capacity to neutralize PAF-induced inflammatory effects, suggesting a potential mechanism by which anti-PC is atheroprotective.

oxLDL, LPC and oxCL can promote the production of factors implicated in inflammation and plaque rupture, and these effects can be inhibited by ANXA5. These findings indicate that these phospholipids have inflammatory properties with direct relevance for plaque rupture and that ANXA5 could be an interesting novel therapy in CVD.

Both SLE cases and SLE controls exhibit low anti-PC levels and this might contribute both to CVD in SLE and to SLE per se. In SLE, the lipoprotein pattern is not characterized by small dense LDL in contrast to conditions like diabetes.

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