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Lyso-PC and macrophage interplay. Scavenging through the fatty side of atherosclerosis

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Lyso-PC and macrophage interplay

Scavenging through the fatty side of atherosclerosis

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Faculty opponent
Professor Germán Camejo
AstraZeneca Cardiovascular Discovery, Mölndal, Sweden

Till mina killar!

When I use a word, it means just what I choose it to mean — *neither more nor less.*

Humpty Dumpty in Through the Looking Glass

Glöm inte att koka disklasen!

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I. Olofsson K.E., Andersson L., Nilsson J., Björkbacka H. Nanomolar concentrations of lysophosphatidylcholine recruit monocytes and induce pro-inflammatory cytokine production in macrophages. *Biochem Biophys Res Commun.* (2008) 370: 348-52.

II. Berg K.E., Gonçalves I., Alterbeck M., Nitulescu M., Edsfeldt A., Persson A., Nilsson M.N., Prehn C., Adamski J., Fredrikson G.N., Nilsson J., Björkbacka H. Lyso-PC promotes a pro-inflammatory monocyte/macrophage phenotype associated with vulnerable human carotid plaque. *Manuscript.*

III. Gonçalves I., Edsfeldt A., Berg K.E., Grufman H., Björkbacka H., Nitulescu M., Persson A., Prehn C., Adamski J., Nilsson J. High levels of lysophosphatidylcholine and lipoprotein-phospholipase A2 are associated to inflammation in human carotid plaques. *Manuscript.*

IV. Berg K.E., Ljungcrantz I., Andersson L., Bryngelsson C., Hedblad B., Fredriksson G.N., Nilsson J., Björkbacka H. Elevated $CD14^{++}CD16^{-}$ monocytes predicts cardiovascular events. *Submitted.*

Published papers not included in thesis

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III. Zhao M., Wigren M., Dunér P., Kolbus D., Olofsson K.E., Björkbacka H., Nilsson J., Fredrikson GN. FcgammaRIIB inhibits the development of atherosclerosis in low-density lipoprotein receptor-deficient mice. *J Immunol.* (2010) 184:2253-60

IV. Teige A., Bockermann R., Hasan M., Olofsson K.E., Liu Y., Issazadeh-Navikas S. CD1d-dependent NKT cells play a protective role in acute and chronic arthritis models by ameliorating antigen-specific Th1 responses. *J Immunol.* (2010) 185:345-56

V. Kolbus D., Ramos OH., Berg K.E., Persson J., Wigren M., Björkbacka H., Fredrikson GN., Nilsson J. CD8+ T cell activation predominate early immune responses to hypercholesterolemia in $Apoe^{-}(/)^{-}$ mice. *BMC Immunol.* (2010) 11:58

VI. Dunér P., To F., Berg K., Alm R., Björkbacka H., Engelbertsen D., Fredrikson GN., Nilsson J., Bengtsson E. Immune responses against aldehyde-modified laminin accelerate atherosclerosis in Apoe-/- mice. *Atherosclerosis.* (2010) 212:457-65

VII. Saxena A., Rauch U., Berg K.E., Andersson L., Hollender L., Carlsson A.-M., Gomez M.F., Hultgårdh Nilsson A., Nilsson J. and Björkbacka H. The Vascular Repair Process after Injury of the Carotid Artery is regulated IL-1RI and MyD88 Signalling. *Cardiovasc. Res.* (2011) *In press*

Submitted papers not included in thesis

I. Edsfeldt A., Dias N., Elmståhl B., Müller M.F., Berg K., Nitulescu M., Persson A., Ekberg O., Gonçalves I. Low carotid calcium score is associated with higher levels of glycosaminoglycans, TNF-alpha and PTH in human carotid plaques. *Submitted to Stroke*

Introduction

Cardiovascular disease causes 41% of all deaths in Sweden and 12% of all Swedes suffer from some form of cardiovascular disease [1]. Worldwide, cardiovascular disease is a growing problem. Several of the major risks factors are increasing in western societies, such as diabetes mellitus, obesity and lack of exercise, and the developing countries are following the same path. Today, there are treatments that decrease the cholesterol levels in blood and reduce the blood pressure. However, this is not enough to prevent the development of disease. In order to improve treatment and enhance survival, we need to expand our knowledge about the cardiovascular disease mechanisms. We need to understand how atherosclerosis starts in the vessel wall and which mediators and cells that are involved, and in which way. We need to be able to identify dangerous rupture-prone plaques, which cause the majority of acute cardiovascular events. We need to pinpoint mechanisms in atherosclerosis, and design accurate treatments to these. There is a lot of work to do!

The past years as a Ph.D. student have been a journey. As in all journeys there have been ups and downs. There has been hard work, joy when things have gone the right way and tears when experiments failed. This journey has thought me a lot about life, not only the life as a scientist but also about the life as a whole. Now, at the end, or at the beginning of my thesis, I will try to give you a chance to be part of my years as a Ph.D. student.

The cardiovascular system

The cardiovascular system is composed of the heart, arteries and veins. In an adult human being, there is around 5 liters of blood moving around in the cardiovascular system. The blood is composed of plasma, red blood cells, white blood cells and platelets. The whole vessel system is composed of almost 97 000 kilometer of vessel; this will take you more than twice around the world. To understand the system failures during the development of atherosclerosis, it is important to understand the normal composition and function of the vessel wall as well as the immune system.

The vascular system

The main function of the vascular systems is the transport and the distribution of cells, nutrients and gases. Arteries are responsible for transport and distribution of nutrients and oxygen to the cells in the body, while veins are responsible for transport of carbon dioxide and waste products from the cells. Cells throughout the body use the vascular system to communicate with cells in other parts of the body; they do so by the release of cytokines and hormones among others. The cells of the immune system use the vascular system as an autobahn to reach parts of the body, which need the immune systems assistance.

Arteries

Arteries are the vessels transporting blood from the heart. The structural composition of the artery wall is composed of three layers namely *tunica intima*, *tunica media*, and *tunica adventitia* (fig.1). *Tunica intima* is the most inner layer closes to the lumen of the vessel and is composed of a continuous single layer of endothelial cells, a layer of connective tissue and a membrane composed of elastic fibers. Underneath the *tunica intima* is *tunica media*, which is composed of elastic tissue and smooth muscle cells. *Tunica media* is separated from the most outer layer the *tunica adventitia*, by the external elastic lamina. *Tunica adventitia* is composed of mostly collagen and is anchoring the vessels to surrounding tissues. The challenge for arteries is the high pressure from the blood. The blood moving in the artery causes stretching of the endothelial layer. This force is termed shear stress and is important for the normal function of the blood vessel [2]. The importance of shear stress in atherosclerosis will be further discussed in the section “Atherosclerosis, from healthy vessel to plaque”.

Veins

Veins are the vessels transporting blood towards the heart. The structural composition of the veins resembles the structural composition of the arteries but the vessel wall of veins lack both the internal and external elastic lamina. Some veins are also equipped with valves, which purpose is to prevent backflow of blood.

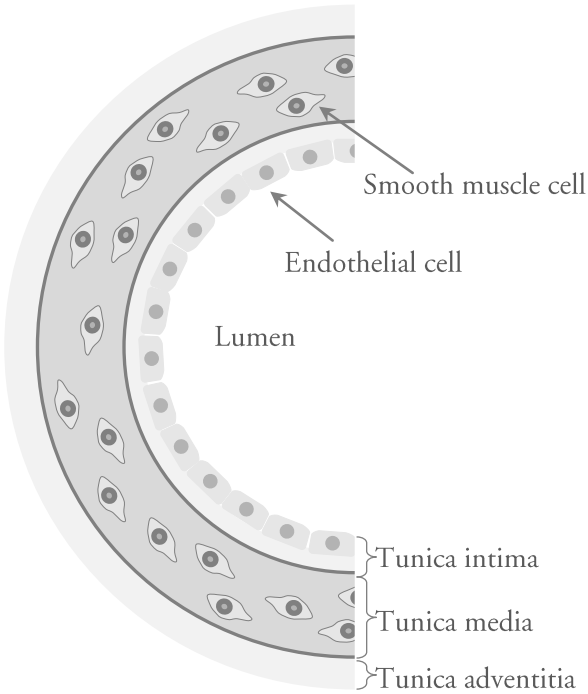

Figure 1. Schematic picture of the artery wall, the three layers and the locations of smooth muscle cells and endothelial cells.

The immune system

Evolution has equipped us with an immune system. The immune systems main function is to respond to and defend us from invading infectious microbes. All living organisms have a defense system, including some of the simplest organisms such as the bacteria. The bacteria possess the ability to respond to and destroy viruses targeting bacteria with a system composed of enzymes [3]. But as the organism complexity increases, a more multifaceted defense system is required. In the section to follow, there will be a brief introduction to the complex human immune system (fig. 2); with a focus on those parts important for understanding this thesis.

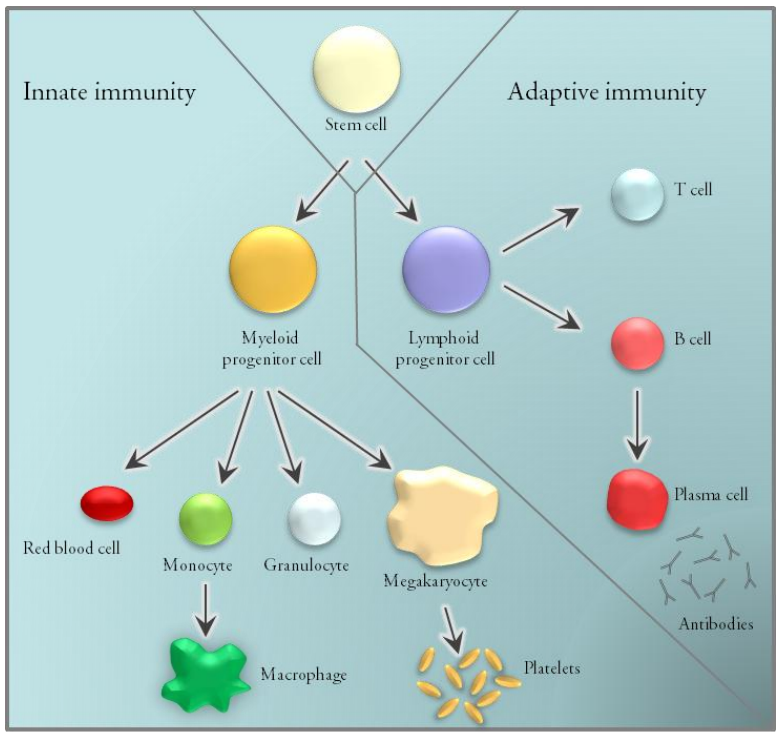

Figure 2. Schematic picture of the human immune system with focus on those parts discussed in the sections to follow.

Innate immunity

The first line of defense is the innate immune system. Innate immunity is mounting a response rapidly and is responsible for directing the adaptive immunity. It is composed of 1) physical and chemical barriers, 2) blood proteins, 3) cytokines, and 4) cells; such as macrophages. Cells of the innate immune system rely on recognition of conserved structures on pathogens; pathogen-associated molecular pattern (PAMP) [4]. For this reason they have evolved receptors called pattern-recognition receptors (PRR). PRR also recognize damage-associated molecular patterns (DAMP), which are endogenous structures produced during stress [5]. Both Toll-like receptors (TLR) and scavenger receptors (ScR) are families of PRR [6]. These receptors will further be discussed later in the section of "Atherosclerosis".

Monocytes

Monocytes are the precursor of macrophages and dendritic cells [7]. They are scavenging through the body, moving around with the help of the vascular system, and constantly looking for signs of invaders. When the monocytes encounter any signs of invaders, they will leave the vascular system and enter the tissue. Until recently, monocytes were considered to be a homogenous population [8]. Today there are at least three accepted subclasses of monocytes separated by expression of the surface markers[1] CD14 and CD16; the classical monocytes ($CD14^{++}CD16^{-}$), the intermediate monocyte ($CD14^{++}CD16^{+}$), and the non-classical monocyte ($CD14^{+}CD16^{+}$) [9]. But not only the expression of CD14 and CD16 separates the monocyte subpopulations [7]. Other surface markers differ as well and this will precondition the monocytes in how they sense the surrounding, and thus how they respond to stimuli. When monocytes enter tissue, they differentiate into macrophages.

Macrophages

Macrophages are the big eaters (from the greek *makros* "large" and *phagein* "eat"). When macrophages encounter cellular debris or microbes, their role is to clear them away by phagocytosis and, if the phagocytized particle is dangerous, alert the adaptive immune system. The macrophage will process and present the phagocytized particle

[1] Surface markers characterize cells and distinguish subpopulations of cells. But surface markers are not there just to characterize the cells. The cells use the surface markers for sensing the surrounding, crosstalk with other cells, and for migration.

as an antigen on major histocompatibility complex[2] class II (MHCII). If the macrophage is recognizing the phagocytized particle as dangerous the presentation of the antigen will be accompanied with expression of membrane bound receptors and release of cytokines[3] (co-stimulation). However, if the particle is considered as harmless, the co-stimulation is absent. The ability to present antigen on MHCII is exclusive for antigen-presenting cells; macrophages, dendritic cells, and B cells. Throughout the body, there are tissue specific stationary macrophages. Depending on their location and the local microenvironment, they will adopt a phenotype specific for the need [7, 10, 11]. However, in the case of inflammation monocytes will migrate in to the tissue and differentiate into classically activated macrophages (M1) or into alternatively activated macrophages (M2)[4] [10]. Macrophages possess a high degree of plasticity as they can change their behavior with changes in the microenvironment [12, 13]. The classically activated macrophages are characterized by release of pro-inflammatory cytokines and mediators, and functionally they are licensed to kill microbes. The alternatively activated macrophages on the other hand, are regulators of inflammation and cleaners, and they produce anti-inflammatory cytokines.

Today, there is no knowledge about how monocyte subpopulations and macrophage phenotypes are connected [10, 14].

Platelets

Platelets are the product of megakaryocyte fragmentation. Platelet's main functions are hemostasis, keeping the blood in the cardiovascular system and initiating mending of ruptured vessels. In the normal situation the endothelial cells produce substances that will inhibit clot formation. However, when the endothelial cell layer is disrupted platelets will become activated by the access to the connective tissue of *tunica intima* or to soluble platelet agonists released by the endothelium. Altogether, this will start a process resulting in clot formation [15, 16]. Activated platelets also release several

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[2] There are two classes of MHC molecules that present antigens to the adaptive immune system. Class I molecules (MHCI) are found on all cells with nucleus, while class II molecules (MHCII) are found exclusively on the cell surface of antigen-presenting cells.

[3] This is called co-stimulation or a secondary signal, which is crucial for activation of the adaptive immune system. It is composed of both membrane bound receptors (CD80, CD86, CD28), and cytokines produced by antigen-presenting cells, which will direct the differentiation of the T cell.

[4] The M1 and M2 is a broad classification and should be considered as two extremes rather than definite classes.

cytokines and growth factors such as IL-1β, RANTES, and PDGF, implicating a role for platelets in inflammation [16].

Adaptive immunity

The adaptive part of the immune system is the part that has the ability to learn and to remember. The cell populations of the adaptive immune system are not the fastest but the most precise. The adaptive part of the immune system is dependent on the innate part for activation through antigen presentation together with co-stimulation.

T cells

T cells mature into naïve T cells in thymus, hence the T in T cells. Their function ranges from being activators and regulators of other cells to direct killing of infected or altered target cell. As for monocytes and macrophages, T cells are a heterogeneous population. T cells are roughly divided into $CD4^+$ T cells and $CD8^+$ T cells.

Naïve $CD4^+$ T helper (h) cells are activated by presentation of an epitope[5] on MHCII by antigen-presenting cells. Depending on the co-stimulation from the antigen-presenting cell, the $CD4^+$ T cell will differentiate into a mature T cell with different functionalities; subpopulations of $CD4^+$ T cells. Th1 cells are pro-inflammatory and producers of cytokines that will elongate the inflammation and Th2 cells are activators of B cells. $CD8^+$ T cells or cytotoxic T cells are activated through recognition of epitopes on MHCI. They are targeting altered cells, such as infected cells or tumor cells, throughout the body.

Regulatory T cells or Tregs are modulators of immune response. There are both $CD4^+$ and $CD8^+$ regulatory T cells. Their main function is to suppress self-reacting T cells and to maintain tolerance.

B cells

B cell's main functions are to produce antibodies and act as an antigen-presenting cell. Some B cells (B1 cells) produce antibodies that recognize conserved structures on microbes, without a preceding infection. These antibodies are referred to as natural antibodies and are part of the innate immunity. In the contrast, B2 cells produce antibodies involved in adaptive immunity. Most B cells need Th2 cells for activation.

[5] Epitope is the fragment of an antigen that is recognized by the immune system.

Memory cells

Both T cells and B cells have the ability to "remember". After an infection and activation of the adaptive immune system some T cells and B cells will become memory cells. These cells will more easily be activated than naïve T and B cells, if they encounter the same antigen once more. The activation of memory cells is not dependent on a secondary signal.

Inflammation

Inflammation is one of the first defense mechanisms and a response to injurious elements. It is characterized by redness, swelling, heat and pain. The inflammation is caused by release of mediators from cells part of the innate immune system that are activated through PRRs.

In the early stages of inflammation, classically activated macrophages are activated by PAMPs and pro-inflammatory cytokines. Cytokines in the early stage of inflammation are produced by cells of the innate immune system. To sustain the inflammation, activation and differentiation of naïve T cells into Th1 cells is most often crucial [11]. Th1 cells also produce pro-inflammatory cytokines, such as IFNγ. IFNγ is needed to maintain the population of activated macrophages. Even though adaptive immunity is dependent on innate immunity in the early phase of inflammation, a collaboration of the two parts is crucial for sustaining the inflammation.

Just as important as to start and sustain the inflammation is the resolution of the inflammation. In inflammation, macrophages are important because they scavenge microbes and cellular debris. If the antigen is not cleared away properly, the T cells will continue to produce pro-inflammatory cytokines and the resolution is impaired. Resolution of the inflammation is a critical step and any errors could result in chronic inflammation.

Cell communication

In order to maintain durability in a complex system such as the human body, the cells within the body need to be able to talk to each other. The communication routes used by cells of the immune system are through soluble mediators and through cell-cell contact.

Soluble mediators

Cytokines are soluble or membrane bound immune modulating mediators, including chemokines, growth factors, and hormones[6] [17]. Cytokines can act in an autocrine, juxtacrine, paracrine, or endocrine manner and can be divided into pro-inflammatory or anti-inflammatory cytokines. In order to respond to a cytokine, the cells need to express receptors for that particular cytokine. However, how cytokines act is depending on cytokine concentration, cell type affected, timing and microenvironment. IFN-γ as an example is considered to be pro-inflammatory, however IFN-γ could under some circumstances have anti-inflammatory properties [18, 19]. Cells also have the ability to enzymatically cleave the receptors, called shedding. Shedding of receptors could neutralize cytokines in order to control the immune response, elongate cytokine half-life or desensitize the cell [17].

Cell-cell contact

Adhesion molecules are proteins located on the surface of cells and are involved in cell-cell or cell-extra cellular matrix binding. In addition to adhesion, they mediate information to the cell about the surrounding.

[6] If hormones should be considered as cytokines is questioned. However, since hormones follow the definition used here for cytokines, they will be included within the cytokines.

Lipoproteins

Lipoproteins are particles containing proteins and lipids. They function as transport vehicles, delivering lipids to cells throughout the body and collect excess cholesterol for elimination in the liver. The lipoprotein system is highly dynamic with lipoproteins changing load with each other, both proteins and lipids. The lipoproteins have a shell of phospholipids, cholesterol and proteins, and a core of triglycerides and cholesteryl esters (fig. 3).

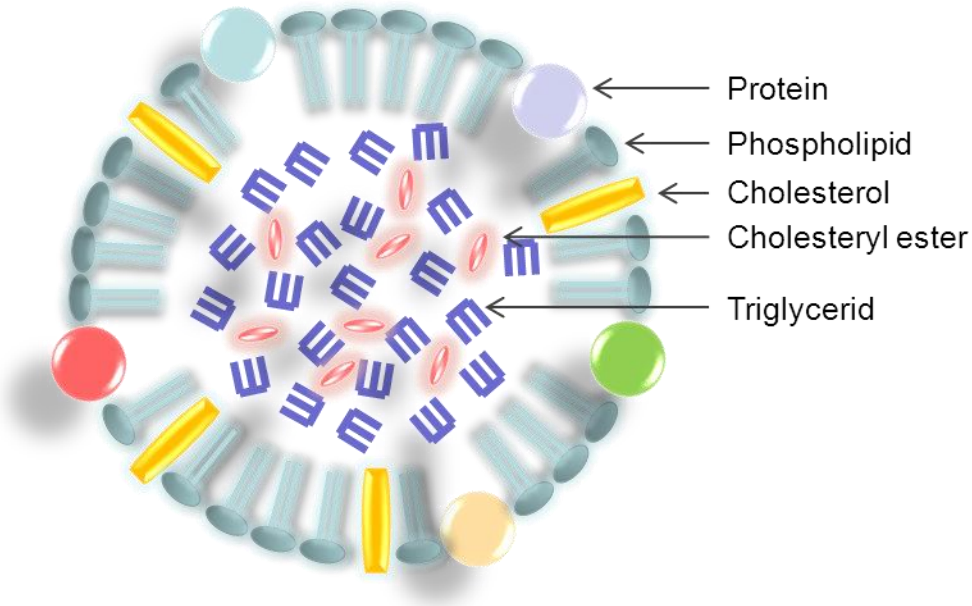

Figure 3. The figure shows a schematic picture of a LDL particle. Cholesteryl esters and triglycerides are in the center of the particle and are surrounded by phospholipids, proteins and cholesterol.

Chylomicrons

Chylomicrons are produced by mucosal epithelial cells in the intestines. Chylomicrons deliver dietary cholesterol and triglycerides to the adipose tissue for storage and to muscle cells for ATP production. The chylomicrons leave the tissue as chylomicron remnants, which are taken up by hepatocytes.

VLDL (very low-density lipoprotein)

In contrast to chylomicrons, the VLDL particle is containing mainly endogenous lipids and is produced in the liver. The VLDL particles deliver triglyceride to the adipose tissue. When the triglyceride level in the particle is lower than the cholesterol level the VLDL particle will be converted into a LDL particle.

LDL (low-density lipoprotein)

LDL has often been misleadingly referred to as the "bad cholesterol"[7]because of the fact that high levels of LDL in blood are associated with atherosclerosis. The main protein in LDL is apolipoprotein B-100 ($ApoB^{100}$), which mediates uptake of LDL by binding to the LDL receptor on cells. LDL is a heterogenic population regarding size [20], where small dense LDL (sdLDL) is the subclass most strongly correlated to atherosclerosis [21, 22]. LDL has a half-life of 3 days [23, 24].

HDL (high-density lipoprotein)

High levels of HDL are associated with low risk of atherosclerosis. HDL is involved in reverse cholesterol transport, where excess cholesterol in cells is exported through ABCA1 or ABCG1 to lipid-poor HDL particles, and transported to the liver. The clearance of cholesterol from lipid laden macrophages and smooth muscle cells by HDL is important in atherosclerosis [25].

[7] This is not in any way a correct assumption. We need the LDL particle for transport of lipids in the circulation.

Atherosclerosis, from healthy vessel to plaque

Atherosclerosis is a disease of large and medium sized arteries. The word atherosclerosis comes from the greek word for *athero* and *sclerosis*. *Athero* means gruel or paste, and *sclerosis* means hardness. Atherosclerosis is a complex multifactorial disease that starts early in life. Already in young people, the first signs can be detected. These so called fatty streaks are composed of lipids and lipid laden cells. In this stage, the disease is reversible. During the years to follow, the fatty streak evolves and a plaque is formed (fig. 4).

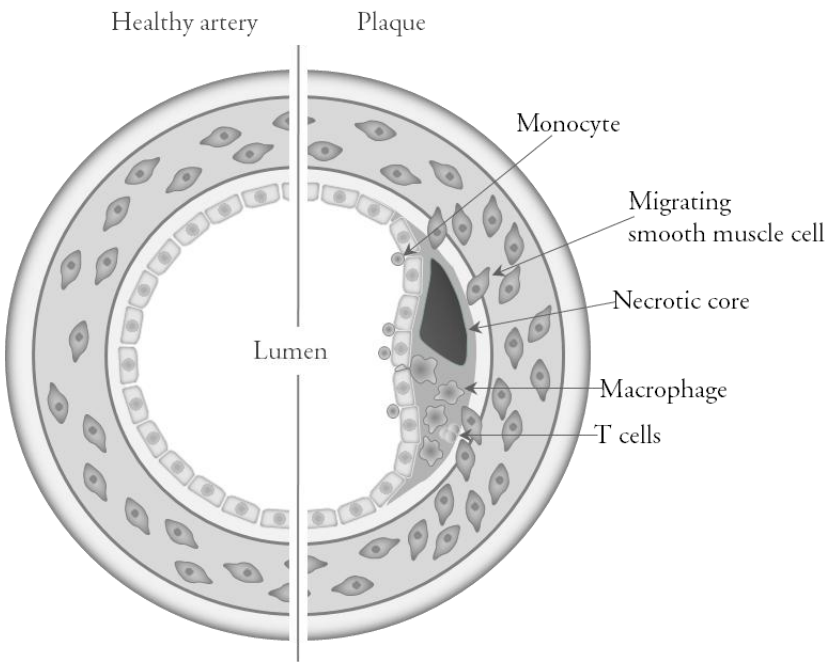

Figure 4.The figure shows a cross-section of an artery. On the right side of the picture, a plaque and the plaque components are illustrated.

Shear stress

Shear stress is the force that acts on the vessel wall, causing stretching of the endothelial layer when blood is moving in the artery. During normal laminar shear stress, endothelial cells adopt an uniform orientation and align in the direction of the blood flow [26]. Shear stress affects the endothelial cells to produce anti-thrombotic factors [2, 27]. Shear stress also inhibits endothelial cell proliferation, keeping the interactions between endothelial cells intact [28]. When blood flow is disturbed, however, the endothelial cells change their normal behavior and those regions exposed to disturbed shear stress are susceptible to plaque initiation. Endothelial cells under low shear stress are polygonal and disarranged [29]. Disrupted shear stress up-regulates adhesion molecules, such as P-selectin, vascular cell adhesion protein 1 and inter-cellular adhesion molecule 1 on endothelial cells [30, 31]. Disrupted shear stress also induces cytokine and chemokine expression, and enhances endothelial cell response to cytokine stimuli [30, 32]. Taken together, up-regulated adhesion molecules, cytokines and chemokines in response to disrupted shear stress mediates monocyte-endothelial cell interactions [30, 33], which is crucial for atherosclerosis initiation. Smooth muscle cells are also affected by shear stress. Smooth muscle cell proliferation and migration are decreased by shear stress [27, 34], two features important in neointima formation[8].

The glycocalyx is a layer of membrane-bound molecules lining the lumen side of the endothelial cell layer. The glycocalyx is thought to function as a permeability regulator and as an endothelial mechano-sensor for shear stress [35]. The molecules in the glycocalyx include proteins, glycolipids, glycoproteins, and proteoglycans [36]. Notable, in regions with disturbed flow the glycocalyx layer is thinner or diminished [37, 38]. Regions with disturbed flow show an increase in permeability properties, with an increase of LDL leakage into the vessel wall [37]. A thinning of the glycocalyx layer mediates leukocyte adhesion to the endothelium [39]. Moreover, the glycocalyx layer is also diminished due to atherogenic risk factors, such as hyperglycemia and hypercholesterolemia [38, 40, 41].

[8] Neointima formation results in a thickening of the intima and can be divided into three steps; 1) smooth muscle cell migration from *tunica media* to *tunica intima*, 2) smooth muscle cell proliferation, and 3) synthesis and deposition of extra cellular matrix. Intimal thickening can result in stenosis (narrowing of the vessel).

Lipids, oxidative and enzymatic modifications

sdLDL generation

If there is a defective clearance or overproduction of VLDL particles in the liver, the plasma level of VLDL rises and thereby an increase of triglyceride levels in plasma. Together with a rise in VLDL and triglyceride levels, the level of LDL will also rise. Above a threshold of 1.5 mmol/l, the LDL level is not increasing [42]. Instead, the LDL particles are just turning smaller and more dense [42]. The LDL particle will exchange cholesterol esters for triglycerides with the VLDL particle, via the cholesterol ester transfer protein mechanism [42, 43]. Hepatic lipase will remove triglycerides from the newly formed triglyceride-rich LDL particle, leaving it smaller and more dense [42]. SdLDL has lower affinity to the LDL receptor than the normal sized LDL particle [44], and will stay in the circulation for a longer time than the normal sized LDL particle [42].

Lipid retention

It has long been known that LDL particles get trapped in intima, preceding atherosclerosis [45, 46]. Based on these early findings, the "Response to retention"[9] hypothesis was formulated [47]. LDL and especially sdLDL has an increased affinity to extra cellular matrix in the vessel wall [48]. Further evidence of proteoglycan binding potential of the LDL particle established the response to retention hypothesis as a key event preceding atherosclerosis [49]. LDL gets trapped in the intima in vessel wall regions with increased trans-endothelial permeability, low shear stress, and a diminished glycocalyx layer. In the intima, the LDL particle will be oxidative and enzymatically modified, resulting in oxidized LDL (oxLDL) [50]. SdLDL has in addition to its increased affinity to extra cellular matrix, been shown to be more prone to become oxidized than larger LDL particles [51]. Retention of LDL is mediated by ApoB binding to extra cellular matrix [52], and is increased with increased oxidation of the LDL particle [53]. During oxidation in the intima, newly formed mediators and adducts – neo-epitopes, elicit immune responses [54, 55]. OxPL and oxPL-protein adducts, MDA modified extra cellular matrix and ApoB, and lyso-PC, just to mention a few of these newly formed mediators and adducts [54, 56, 57].

Phospholipase A_2 family

Enzymes in the phospholipases A_2 (PLA_2) family hydrolyze the ester bond at the sn-2 position on fatty acids. The PLA_2 family can be divided into two main categories. The

[9] Response to retention hypothesis states that the subendothelial retention of lipoproteins is a key event and an absolute requirement for the pathogenic process leading to atherosclerosis.

first category includes all cytosolic PLA_2, and the second category includes extracellular PLA_2. Lipoprotein-associated PLA_2 and secreted PLA_2 constitute the extracellular PLA_2 category and have been implicated in the pathophysiology of atherosclerosis [58, 59]. Lp-PLA_2 is specific for the substrates oxPL and PAF, leaving non-oxidized phospholipids unmodified [60]. Lp-PLA_2 is also called platelet-activating factor acetylhydrolase (PAF-AH) and is mainly associated with the LDL particle. Among the different LDL species, Lp-PLA_2 is predominantly associated with sdLDL [61, 62]. In plaque, Lp-PLA_2 can be synthesized by macrophages, T cells, and mast cells in addition to being brought in by lipoproteins [63]. Lp-PLA_2 is expressed in large quantities in the necrotic core of human plaques [64]. A meta-analysis of 32 prospective studies with 79 036 patients, showed that both Lp-PLA_2 activity and mass were associated with pro-atherogenic lipids, vascular risk, and each other [65].

When Lp-PLA_2 hydrolyzes the ester bond on oxPL, lyso-PC will be formed (fig. 5).

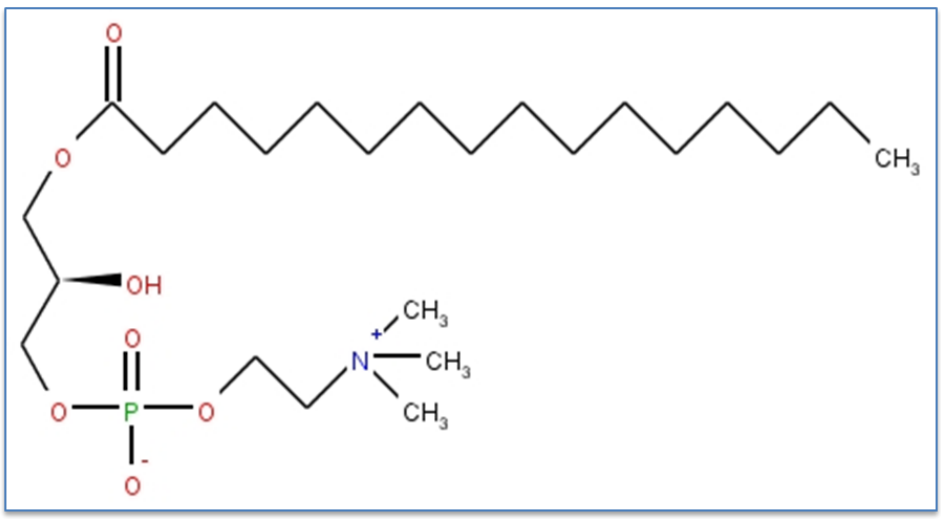

Figure 5. The molecular structure of lyso-PC 16:0, an enzymatic product after hydrolyzes of oxidized phospholipids by Lp-PLA_2.

There are several different lyso-PC species, with lyso-PC 16:0 being the most frequently occurring in LDL [66, 67]. It has long been known that Lp-PLA_2 correlates positively with higher lyso-PC production during oxidation of LDL particles [61]. Lyso-PC is more predominantly found in sdLDL compared to large buoyant LDL particles [63]. Lyso-PC will be further discussed in the section "Result and discussion".

There are other products formed by Lp-PLA_2. One of them, namely oxNEFA (oxidize non-esterified fatty acids) is highly associated with inflammation and atherosclerosis

[68]. But Lp-PLA_2 can also give rise to less inflammatory products as it cleaves PAF into lyso-PAF and acetate, render them biologically inactive [69, 70]. However, Lp-PLA_2 has also been proposed to have a role in activation of lyso-PAF through trans-acetylation [71].

Cells involved in inflammation in the vessel wall

As mentioned previously, the immune system's main function is to respond to and defend us from infectious microbes. However, the immune system can in some cases respond to noninfectious substances, foreign or self-produced. In the case of responding to self, the response could result in an autoimmune disease. This is what occurs in atherosclerosis [72]. Both vascular wall resident cells and cells migrating in from adjacent tissues, contribute to the plaque development.

Endothelial cell

Endothelial cells are the gate keepers of the plaque. In addition to the effect of shear stress on endothelial cells already mentioned, oxLDL has an impact on endothelial cells. OxLDL down-regulates ABCA1 expression on endothelial cells, inhibiting reverse cholesterol transport [73]. Modified LDL also induces pro-inflammatory cytokine release by endothelial cells [74], and up-regulate adhesion molecules [75, 76]. All the above mentioned features have implication in the early plaque development.

Smooth muscle cell

In the beginning of plaque development, smooth muscle cells are migrating from the *tunic media* into the *tunica intima*. In *tunica media*, the smooth muscle cells possess a contractile phenotype while smooth muscle cells in *tunica intima* possess a synthetic phenotype [77, 78]. The synthetic smooth muscle cells have enhanced uptake of lipoproteins [79]. Synthetic smooth muscle cells are prone to proliferate, an ability that is not present in the contractile smooth muscle cell repertoire [78]. The synthetic smooth muscle cell is also involved in stabilization of the plaque and can synthesize 25 to 46 times more collagen than the contractile smooth muscle cell [80]. Smooth muscle cells have been somewhat forgotten in the induction of immune responses but have been shown to release both cytokines and up-regulate adhesion molecules [78, 80]. Implicating a possible role for smooth muscle cells in monocyte migration and entrapment of cells within the plaque.

Monocytes and macrophages

As mentioned earlier, there are three subpopulations of monocytes in blood. How these populations correlate with plaque macrophages is unknown [14]. It has been shown, however, that the population of $CD14^{++}CD16^{+}$ monocytes is associated to

cardiovascular events, in high risk patients suffering from chronic kidney disease [81, 82]. As soon as monocytes enter tissue, they differentiate into macrophages. If macrophages express CD14, it could have implications for the uptake of mmLDL by macrophages since CD14 together with TLR4 is required in this process [83]. Moreover, mutations attenuating TLR4 signaling in humans are associated with lower risk to developing atherosclerosis [84], and a defect in the signaling pathway of most TLRs in mice prone to atherosclerosis results in a decrease of atherosclerosis development [85]. In addition to TLR4, TLR2 is also found in areas prone to plaque development and has been shown to recognize modified lipids [86]. When the LDL particle has been extensively oxidized the particle is no longer recognized by either LDL receptors or TLRs, instead ScRs recognize oxLDL and mediates its uptake [87]. The ScRs SR-A and CD36 seem to account for up to 90% of the uptake of modified LDL by macrophages, however, several other ScRs are proposed to be involved [87]. Macrophages in plaque are heterogeneous and can be divided into classically activated and alternatively activated macrophages. Classically activated macrophages are the producers of type 1 cytokines, cytokines with a pro-inflammatory capacity. In contrast, alternatively activated macrophages are the reparatory macrophages with high expression of ScRs. However, their precise role in atherosclerosis has not yet been completely elucidated. In the plaque, several inducers of macrophage apoptosis are present. These inducers include high concentrations of pro-inflammatory cytokines, ER stressors, and producers of oxidative stress [88]. Macrophage death[10] induces expansion of necrotic core[11], leading to vulnerable plaque formation if the clearance of apoptotic cells is impaired [88, 89]. OxLDL has been shown to inhibit clearance of apoptotic cells [87].

Foam cells

It has long been known that smooth muscle cells contribute to the formation of a foam cell population [78]. Dendritic cells could potentially also contribute to foam cell formation [90]. But, the majority of the foam cells however are of macrophage origin. Foam cells are cells which have been taking up extra cellular lipoproteins in the plaque by endocytosis. The death of foam cells are contributing to progression of the disease [91].

[10] Macrophage death includes both apoptosis and necrosis. Apoptotic cells not cleared away progresses into secondary necrotic cells.

[11] Necrotic core is often called lipid core because this area contains large amount of extracellular lipids and cellular debris.

T cells

In humans, around 20 % of the cell population within the cap of an atherosclerotic plaque are T cells, whereas in other regions of the plaque T cells are found more sporadic [92].

Th1 cells are the main producer of INFγ and the dominating T-cell subpopulation in the plaque [93]. It has been shown that there are T-cell clones in human plaques that upon stimulation with oxLDL proliferate and produce IFNγ [55]. IFNγ production contributes to sustained inflammation in the plaque, by enhanced recruitment of T cells and macrophages, activation of antigen-presenting cells, and inhibit formation of foam cells to mention some [94]. IFNγ also prevent infiltration and proliferation of smooth muscle cell [94], and impair synthesis of new collagen by smooth muscle cells [94, 95].

Th2 cells are the activators of B cells and produce the cytokine IL-4. Th2 cells have been addressed an protective role in atherosclerosis [93]. However, their role in atherosclerosis is controversial [96].

The regulatory T-cell population in atherosclerosis has been proposed to have a protective role due to their ability to suppress Th1 response. Regulatory T cells can be found in all stages of atherosclerosis and constitute 0.5-5% of the total T-cell population in a plaque, and are increased with plaque severity [97]. In patients with acute coronary syndrome, the peripheral regulatory T cells and their ability to suppress responder cells was shown to be reduced compared to healthy controls and patients with stable angina [98]. However, regulatory T cells in atherosclerotic plaque is scarce in comparison with other chronic conditions such as eczema or psoriasis with 15-25% regulatory T cells in the inflamed skin lesions [97].

$CD8^+$ T cells constitute ~30% of the T cells found in human lesions [99], and $CD8^+$ T cells increase with disease severity up to 50% of the total lymphocyte population [100]. $CD8^+$ T cells recognize antigen through MHCI, and oxLDL specific $CD8^+$ T cells could potentially target and kill cells that has taken up oxLDL in the plaque [101].

B cells

B cells have been ascribed both pro- and anti-inflammatory properties in atherosclerosis and can be a part of either the adaptive or the innate immune system. As a part of the innate immune system, B1 cells produce natural antibodies towards oxLDL neo-epitopes [102]. These antibodies recognize oxPL and can be found in atherosclerotic lesions [102]. However, there are also antibodies belonging to the adaptive immune system that recognize other atherosclerotic neo-epitopes. There are both IgM and IgG towards modified $ApoB^{100}$ neo-epitopes in circulation, which also correlates to plaque structure [103]. Depleting B2 cells with CD20 antibodies in atherosclerosis prone mice reduces atherosclerosis, suggesting that the natural

antibodies could be protective while adaptive B-cell response could be atherogenic [104, 105].

Stable vs. rupture prone plaque

Plaques can exhibit various sizes and various contents. They are like snowflakes, no two are the same. But they can be put into categories, depending on plaque consistence. There are some that have a lipid-rich necrotic core with dying macrophages, thin fibrous cap and a high degree of inflammation, that are termed thin-cap fibroatheroma [106, 107]. Others have a thick fibrous cap and are rich in smooth muscle cells and collagen. The latter could give rise to symptoms and life-threatening conditions by stenosis. However, the most dangerous plaques are the ones among the thin-cap fibroatheroma plaques that are prone to rupture [106, 107].

The plaques vulnerability is determined by three major plaque features: 1. the size and the consistency of the core, 2. the thickness of the cap, and 3. the inflammation and repair within the cap [108].

Plaque rupture

There are two possible events leading to rupture; fibrous cap rupture or endothelial erosion [107]. Both possibilities results in exposure of plaque content, such as collagen and tissue factor, to cells in the blood. This initiates activation of platelets and induce a cascade to prevent bleeding and start vessel repair by formation of a clot[12]. If the thrombus or a part of the thrombus breaks free, it can cause narrowing or occlusion in other parts of the vascular system[13]. It is estimated that around 90% of an acute thrombus in carotid is due to cap rupture while the other 10% is due to endothelial erosion [109].

Silent rupture

Not all ruptured plaque results in symptoms. Some asymptomatic plaques rupture repeatedly, causing repair mechanisms with proliferating smooth muscle cells resulting in stenosis without causing symptoms. These ruptures are called silent ruptures. In persons dead from non-cardiac causes, 9% had silent ruptures [110].

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[12] The formation of a clot is called thrombosis while the clot itself is called a thrombus.

[13] A thrombus that causes occlusion in other parts of the body is called emboli.

Catabasis and regression of the plaque

In the plaque, a dysregulated resolution of inflammation and return to the normal steady state is impaired. To resolve the inflammation (catabasis) and clear away the plaque components from the intima, some key events are more important than others.

Inhibition of inflammatory cell recruitment and inflammation

In order to inhibit recruitment of cells involved in the inflammation, adhesion molecules on endothelial cells needs to be down-regulated. Certain stop signals such as lipoxins, resolvins, and some prostaglandins induce migration of monocytes specialized in non-phlogistic[14] phagocytosis of apoptotic cells [111, 112]. These macrophages are most likely of the alternatively activated macrophage subpopulation [113]. A shift from the classically activated macrophages to alternatively activated macrophages in the plaque will also affect the cytokine profile in the plaque, and probably also induce a shift from pro-inflammatory cytokines to anti-inflammatory cytokines. Anti-inflammatory cytokines, such as IL-10 and TGF-β , mediate suppression of T-cell activation, inhibit pro-inflammatory cytokine production, enhance efferocytosis, and induce collagen production [113]. Macrophages of a certain subpopulation are not static. With changes in the microenvironment macrophages are able to transform from being a participant in the inflammation to a participant in the catabasis [13].

Efferocytosis

Efferocytosis is the phagocytic clearance of apoptotic cells. In advanced plaque, efferocytosis is impaired, which results in apoptotic cells becoming post-apoptotic or secondary necrotic [114]. Post-apoptotic and secondary necrotic cells contributes to prolongation of the inflammation [113]. Functional efferocytosis also inhibits pro-inflammatory cytokine production [114].

Cell egression

It is not enough to inhibit recruitment of cells to resolve inflammation in the plaque; cell egress also needs to be induced. During plaque regression there is cell egress both through lymphatic vessels and through endothelial cells back out into the aortic vessel [115]. However, under conditions favoring atherosclerosis there is a reduced egression of the immune cells, resulting in an entrapment of cells within the plaque [116].

[14] Phlogistic means inflamed.

The recipe for a stable plaque

Lifestyle or genes

Both lifestyle and genes will contribute to the development and the outcome of the complex multifactorial disease atherosclerosis.

The metabolic syndrome

The metabolic syndrome is referring to several factors contributing to increased risk of developing cardiovascular disease and diabetes mellitus. Criteria involve obesity, disturbance of blood cholesterol and glucose levels, and high blood pressure. Several of the risk factors are possible to modify but some are not changeable.

Non-modifiable factors

There are three non-modifiable factors; genes, gender, and age.

Genes

Genetic factors causing atherosclerosis comprises 30-60% of the cases [117]. Today, 17-25 loci have been identified to be associated to cardiovascular disease [117, 118], and several more is thought to be involved. Identified genes could contribute to the understanding of the initiation and the progression of cardiovascular disease, contribute to the assessments of the atherosclerotic burden, and contribute to identifications of new therapeutic targets in cardiovascular disease.

Gender

Several factors contribute to the differences between men and women regarding the metabolic syndrome where women have lower incidence of cardiovascular disease compared to age-matched men [119]. Several of the gender-specific beneficial influences on atherosclerosis can be addressed to estrogen. Estrogen lowers the activity of hepatic lipase, giving men twice as high hepatic lipase activity with an increased formation of sdLDL [42, 120]. In women after menopause, however, the differences in sdLDL seen between premenopausal women and men is abolished [120]. Estrogen inhibits vascular smooth muscle cell proliferation in a co-culture with endothelial cells [121], and has a positive influence on hypertension [122]. Estrogen also has a positive impact on fat distribution and body weight in premenopausal women [119]. These

features are just some examples of the beneficial influence of estrogen on cardiovascular disease.

Age

Age is an independent risk factor for cardiovascular disease. With age, the artery wall gets stiffer and there will be abrasion damage to the vessel wall [123]. The immune system is changing over time with an up-regulation of the inflammatory response. In elderly, the immune system is challenged with diseases that is more frequent in later stages of life such as tumors, autoimmunity, and inflammatory chronic diseases [124]. Age has also an impact on lipid levels in blood. In both men and women, there is an increase in LDL levels after the age of 20 years and in men HDL levels decrease during puberty and early adulthood [125].

Modifiable factors

Several of the factors that could be modified are highly connected with other modifiable and non-modifiable factors.

Hypertension

Hypertension is a stress factor for the heart, the immune system and the vascular system. Hypertension is associated with increase in inflammatory markers; hypertension could however, also be a result from systemic inflammation [126]. Smoking, obesity, age, physical inactivity, alcohol consumption, gender, and stress, are some of the factors contributing to hypertension [126, 127].

Dyslipidemia

In a normolipidemic person, food has a great impact on lipoprotein phenotype. Even though a high total cholesterol level in blood is associated with high risk of cardiovascular disease, the intake of fat is crucial for our wellbeing and has been proven to be beneficial for our lipoprotein profile in blood [128, 129]. A diet high in fat induces lipoprotein lipase activity resulting in larger LDL particles compared to a diet low in fat and high in carbohydrates which induces an increase of sdLDL [129, 130]. Moreover, a low fat diet decreases total blood cholesterol levels but it also decreases the level of HDL [128]. In addition, it has been shown that fibrates lower VLDL levels in blood [42].

Obesity

Obesity is highly associated to other risk factors such as high blood pressure, dyslipidemia, and diabetes mellitus [131]. Obesity is also associated with cardiovascular mortality [131]. Visceral fat or abdominal fat is more common in men compared to women and is a factor of the metabolic syndrome. Visceral fat is an

independent risk factor even in a normal range of BMI [131]. In men, visceral fat is associated with coronary heart disease [132]. Adipose tissue also contributes to systemic inflammation with an increase in the pro-inflammatory cytokines TNF-α and IL-6 [131].

Physical inactivity

Physical activity decreases and postpones cardiovascular disease and contributes to an improved lipoprotein profile, glucose hemostasis, insulin sensitivity, and endothelial function [133]. Physical activity also reduces systemic inflammation, visceral fat, and the risk for thrombosis [133]. Everyday physical activity for 30 minutes is recommended to reduce the risk for cardiovascular disease [1].

Diabetes mellitus

The most common cause of death for people with diabetes mellitus is vascular complications [134]. Diabetes mellitus enhance the oxidative stress on the vessel wall, and contributes to increased inflammation and thrombosis [135-137]. People with diabetes mellitus also have an unfavorable lipoprotein phenotype with an increased level of triglycerides, low level of HDL and an increase in sdLDL [138]. Women with diabetes mellitus lose their beneficial influence of estrogen on atherosclerosis development [134].

Smoking

Smoking is the modifiable risk factor most strongly associated with atherosclerosis [139]. Both active and passive smoking contributes to inflammation, lipid oxidation, and endothelial dysfunction [139, 140]. Smoking also has an impact on the the lipoprotein profile in blood inducing higher levels of triglycerides and VLDL and lower levels of HDL [141].

Treatments

As our knowledge about cardiovascular disease increases, new therapeutic targets are found [89]. Today, there are a limited number of therapeutic agents directed towards a few atherogenic targets. These therapeutic agents, statins and aspirin, are usually well tolerated but not all patients are able to use them due to their side effects or the patient's medical resistance. The main focus of treatment today is on more advanced stages of atherosclerosis, however, with increased knowledge and with a more advanced pinpointing of atherosclerotic risk factors and atherogenic mechanisms, targeting of earlier stages in atherosclerosis could be identified.

Statins

Statins are inhibitors of HMG-CoA reductase[15], an enzyme produced in hepatocytes. Statins have been assigned several modulatory functions in atherosclerosis, such as inhibition of cholesterol biosynthesis, inhibition of LDL secretion, inhibition of LDL oxidation, inhibition of accumulation of esterified cholesterol into macrophages, inhibition of inflammation and of ScR expression, increase in eNOS activity, and an increase of uptake and degradation of LDL in the liver [142]. Statins has also been shown to reduces sdLDL levels [42]. Statins reduced all-cause mortality with 15% in both men and women, independent of blood pressure and diabetes [143].

Aspirin or acetylsalicylic acid

Low doses of aspirin are recommended for both primary and secondary preventions of myocardial infarction, stroke, and unstable angina [112]. Aspirins has an ability to inhibit coagulation and inhibit inflammation [112]. Aspirin reduces cardiovascular disease [144], however, not all patients respond to aspirin treatments. Clinical aspirin resistance is found in 60-80% of patients with cardiovascular disease [145]. Prevention with aspirin seems to have a gender specific benefit favoring males [146].

Lp-PLA_2 inhibitor

Darapladib is an Lp-PLA_2 specific inhibitor. Darapladib is in a second large-scale phase III clinical trial to evaluate long-term treatment. Darapladib has been shown to

[15] HMG-CoA reductase is a rate-limiting enzyme in the cholesterol synthesis.

halter necrotic core expansion in 152 patients receiving darapladib compared to 155 patients receiving placebo [64]. After darapladib treatment in pigs, there was a reduction in plaque content of several lyso-PC species compared to control pigs [147]. Pigs treated with darapladib also displayed a reduction in both plasma and plaque Lp-PLA_2 activity, a reduction in plaque macrophage content and a reduced necrotic core [147]. Altogether, treatment with darapladib could stabilize the plaque.

Methodology

Clinical studies

Carotid Plaque Imaging Project (CPIP), (paper II and III)

In the end of 2005 the first patient was included in the CPIP study. Today, March 2011, a total of 511 patients and 465 human carotid plaques have been included into the study. CPIP was designed to identify plaque with an increased risk for rupture and to study mechanisms in human atherosclerosis. Symptomatic patients with ipsilateral symptom (TIA, stroke, or amaurosis fugax) or stenosis greater than 70%, and asymptomatic patients with stenosis greater than 80% had their plaques surgically removed with carotid endarterectomy at Malmö University Hospital. Cardiovascular risk factors were recorded including heredity, hypertension (systolic blood pressure > 140mm Hg), diabetes, coronary artery disease, smoking (in the past or current), and fasting lipoproteins (total cholesterol, HDL cholesterol, LDL cholesterol, and triglycerides). The use of medications such as anti-hypertensive drugs, diabetes treatment, and statins were recorded. Mononuclear leukocytes, granulocytes, erythrocytes, plasma, and serum were collected and stored in liquid nitrogen.

In paper II, a total of 40 patients were included, whereof 20 patients were symptomatic and 20 patients were asymptomatic. Plaques from symptomatic patients were collected within a month from the first symptom. In paper III, a total of 162 patients were included, whereof 91 were symptomatic patients and 71 were asymptomatic patients.

Malmö Diet and Cancer study, (paper IV)

Between 1991 and 1996, 74 138 individuals were invited from the population of Malmö to participate in the Malmö Diet and Cancer (MDC) study. The MDC study is a prospective cohort study that was originally designed to clarify if a western diet is associated with certain forms of cancer, and if oxidative stress and the activity in DNA repairing system could influence the impact of diet on the development of all or certain forms of cancer [148]. When the baseline examination closed in 1996 a total of 28 098 men and women between the ages of 45-73 had complete datasets. From the MDC cohort, 6103 individuals were selected during 1991 and 1994 to constitute a subgroup, the cardiovascular arm, for a more detailed study on cardiovascular risk. Among these, 700 individuals were randomly selected for our

study. Until follow-up at December 2008, 150 out of 700 randomly selected individuals, suffered from cardiovascular events based on information obtained from the Swedish Discharge Registry, the Stroke Register of Malmö, and the Cause of Death Register of Sweden. Eleven individuals from the 150 cases were excluded from further analysis due to subarachnoid or intracerebral bleeding[16].

During baseline investigations, health characteristics were collected. These characteristics were based on clinical health examination and from a self-administered questionnaire. The questionnaire included questions about heredity, occupation, social-economic status, physical activity, previous disease, medication and non-prescript drugs, and diet. Erythrocytes, granulocytes, mononuclear leukocytes, plasma, and serum were collected and stored in a bio-bank. Physical examination included measurement of blood pressure, weight, height, body mass index (BMI), waist circumference, and intima-media thickness (IMT).

Cell cultures

In all cell cultures, independent of primary or secondary cells, the cells have been taken from its natural environment into an artificially created environment. We set the conditions for the cells; manipulate the microenvironment and include or exclude cells and stimuli. Of course this will be reflected on the results obtained from such experiments and all data should be interpreted with caution. Instead of the absolute truth the data should most probably be considered as indicators that support or reject the hypothesis. Data can also give an indication of what we shall measure *in vivo*. In our cell experiments, we use cells with different origin, both primary and secondary cells.

Primary cells

Primary cells are referring to cells taken from living tissue and used for *ex vivo* culturing. Primary cells have a limited lifespan and they cannot be cultured in infinity.

Buffy coats are concentrates of white blood cells and platelets. In paper II and III, mononuclear leukocytes were isolated from human buffy coats and used for lyso-PC and mmLDL stimulation. In paper II, $CD14^+$ monocytes from the mononuclear leukocyte population were isolated with magnetic beads and were stimulated with growth factors to differentiate into macrophages.

[16] Subarachnoid or intracerebral bleeding could be a result of other factors than atherosclerosis.

Bone-marrow derived macrophages were obtained from the mouse femur and tibia. Bone-marrow cells were stimulated with growth factors to resemble macrophages and used in paper I.

Peritoneal macrophages were collected with PBS flushing of the peritoneum. These macrophages were stimulated with lyso-PC in paper I and used as a control for the macrophage-like cell line RAW264.7.

Secondary cells

Secondary cells refer to cells that in some way have been immortalized, either through random mutation or by manipulation. However, even if a cell line is immortalized it should be noted that some cell lines change characteristics after a number of passages or with the change in culturing procedures, and the cell line will no longer be comparable with the original cell line.

RAW264.7 cells are a murine macrophage-like cell line that was established from a peritoneal tumor induced by a virus [149]. In paper I, we use this cell line as a read out for lyso-PC stimulation. MIP-2 transcription and release was measured. RAW264.7 cells were also used for measurements of lyso-PC cytotoxicity.

bEND.3 cells are an adherent murine endothelial cell line. Brain endothelial cells were immortalized with retrovirus infection [150]. In paper I, bEND.3 cells were stimulated with lyso-PC and MIP-2 release was measured.

THP-1 cells are a human acute monocytic leukemia cell line that was established from blood from a boy with acute monocytic leukemia [151]. THP-1 cells have distinct monocytic markers and are commonly used both as monocyte-like cells and after differentiation with growth factors as macrophages. In paper I, THP-1 cells were used in a transmigration assay with lyso-PC as a chemotactic agent.

Methods

As for all the methods used throughout the thesis, the result only reflects the conditions at that given moment when the measurement was done.

Flow cytometry

This technique is used in paper II and IV for counting and characterizing cells. Cells in single cell suspension can be labeled with antibodies directed towards selected antigens. The antibodies used for labeling are conjugated with fluorochromes that can separately be detected in the flow cytometer. To measure individual cells in the flow cytometer, the cells need to be in a single file when passing through one or more

beams of light. Light that is scattered in the forward (FSC) direction is providing information about the size of the cell[17]. Light scattered to the sides (SSC) is measuring the cells granularity[18]. A combination of FSC and SSC gives us the opportunity to distingue different cell types in a heterogeneous cell population. Adding on to the multiparametric analysis of the cell characteristics is the additional measurements by fluorochrome conjugated antibodies. Different fluorochromes have individual excitation and emission wavelength and are detected by separate detectors. However, some if not all fluorochromes have wide emission spectra that will be partly registered by other detectors. This leakage of signal will affect the analysis. By compensation with single labeled control cells, some of the leakage can be reduced. In this thesis fluorescent minus one (FMO) samples have been used when possible, to further minimize the impact of leakage. FMO samples are control samples were all fluorocrome conjugated antibodies without one is used to set the threshold for positive staining of the missing fluorochrome. The flow cytometer (Cyan ADP) used in this thesis can detect FSC, SSC, and 9 additional parameter.

Transmigration assay

THP-1 cells were used in paper I to measure lyso-PCs potential as a chemoattractant. Cells are labeled with fluorescent dye and place on top of a filter with pores. In the bottom reservoir, chemoattractant is added. The filter is placed on top of the reservoir and cells are allowed to transmigrate. The fluorescence of the transmigrated cells is measured from the bottom reservoir and a chemotactic index can be calculated by induced migration divided by spontaneously migrated cells i.e. cells migrated towards medium without chemoattractant. Calculation of an index will allow comparisons of experiments preformed at different time points.

Western blot

To visualize Lp-PLA_2 in LDL obtained from healthy blood donors, western blot was used in paper III. Western blot is a technique that detects specific proteins in a tissue sample or a sample with more than one protein. The proteins in the sample are first separated on a gel according to size. After separation the proteins are transferred to a membrane and the antigen is detected with antigen specific antibodies.

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[17] Cellular debris and living cells, and cells with different size can be distinguished with FSC.

[18] The granularity is dependent on the structure of the cell membrane, the nucleus, and the amount and type of granules.

Enzyme-linked immunosorbent assay (ELISA)

Cytokine release (paper I and III) and Lp-PLA_2 mass (paper II and III) were measured with ELISA. ELISA is an antibody based detection system used for detection of antigens and antibodies. For the detection of antigen a sandwich ELISA is performed. An antibody (capturing antibody) specific for the antigen is bound to a surface. Non-specific binding sites are blocked and solution with an unknown amount of antigen is added. A second antibody (detection antibody) with specificity for a different epitope on the same antigen as the capturing antibody is added. The detection antibody is conjugated with an enzyme. The enzyme is inducing a color change in an added chemical compound. In between every step, the plate is washed for the removal of un-bound antibodies and antigens. The proportional intensity of the enzymatically produced color can be measured.

Luminex

Luminex is a combination of ELISA and flow cytometry. In paper II and III, luminex is used to measure cytokine and chemokine content in medium from cell cultures, in plasma, and in plaque homogenate. Luminex is based on a technology, where dyed microspheres are coated with capturing antibodies towards the selected analyte[19]. A detection antibody conjugated to a fluorochrome, towards the analyte is added. As in the flow cytometer, microspheres are line up into a single file and a light source excites both the dyed microsphere and antigen captured fluorochrome. Detectors are registering the emission wavelength from both the microsphere dye and the fluorochrome at the same time. With this technology, up to 100 different analytes can be detected in the same sample. The main advantages with the multiplex assay compared to ELISA are that multiplex assay is reducing working time and reduces the amount of sample needed for the measurement.

Real time quantitative polymerase chain reaction (q-PCR)

In paper I, q-PCR was used to amplify and quantify MIP-2 messenger RNA (mRNA). Reversed transcriptase converts mRNA into complementary DNA (cDNA). By the use of complementary primers to the cDNA sequence and DNA polymerase, the cDNA can be amplified and quantified.

Histology and homogenization of human carotid plaques

Plaques collected by carotid endarterectomy were snap-frozen in liquid nitrogen immediately after surgery at the operating room. A 1 mm fragment from the most

[19] An analyte is a compound that is detected in an analytic experiment.

stenotic region was used for histology and a 1 mm fragment was used for homogenization.

Plaque fragments were sectioned and used in paper II and III to assess content of lipids, collagen content, calcified areas[20], total plaque area, amount of macrophages (CD68), amount of CCR7 and CD163 staining. The sections were fixed and stained with antibodies or dyes towards the specific targets.

Cytokine levels, Lp-PLA_2 mass, and lyso-PC levels was measured in plaque homogenate in paper II and III.

Intima media thickness (IMT)

IMT in the MDC study states the thickness of the artery wall with ultrasound and was measured in the right common artery. IMT gives an indication of the plaque size.

Lyso-PC measurement

In paper II and III, lyso-PC content was measured in plaque homogenate with tandem mass spectrometry [152]. Metabolites are extracted and diluted in mass spectrometry running solvent and metabolite content is analyzed in a mass spectrometer. In the mass spectrometer, the sample is vaporized and ionized, and separated according to size. The ions are detected and the data are processed into a mass spectrum.

Viability assay

In paper I and II, viability assay where performed to exclude the possibility that the effect of lyso-PC were due to an effect of the cytotoxic potential by lyso-PC. Dying cells lose their membrane integrity and when they do so, lactate dehydrogenase (LDH) is leaking out into the media. The assay uses the LDH ability to convert lactate into pyruvate and NADH through an enzymatic reaction. NADH is converting a colorless added compound into a yellowish color. The intensity of the color correlates with the number of dead or dying cells and can be measured with a plate reader. LDH viability assay measures both cell deaths from apoptotic and from necrotic cells.

[20] During the processing of the sections, the calcified areas will appear as holes in the plaque sections. The area of the holes are measured and referred to as calcified areas.

Statistics

Statistics are used to mathematically show the probability of the same outcome if the exact same experimental setup was repeated. In order to choose the right statistical method a few characteristics of the variables needs to be set. The variables in one group can either be normally distributed or skewed, this means that variables either follow the Gaussian distribution or not. The Gaussian distribution gives rise to a bell shaped graph when plotting the variables and is describing how evenly the variables cluster around the geometric mean[21]. If the columns of data are matched then a paired test should be chosen, and consequently an unpaired test should be used if the data is not matched. Another factor deciding which test to use is the number of variables. Some statistical test will not be reliable if the number variables are too small.

Student's t-test is used in paper I, II, and III to calculate the difference between two normally distributed groups.

Mann-Whitney U test is ranking the variables from two unpaired groups independent of which group the variable originates from. The sum of the ranks in each group is calculated and the difference between the two groups is compared. Mann-Whitney U test was used in paper II, III, and IV.

$\chi 2$ or *chi-square* asses variables on a nominal scale[22] and is used in paper IV.

Kaplan-Meier estimator calculates the probability of survival from life-time data. Kaplan-Meier is used in paper IV.

Linear regression fits a linear slope through the plotted variables based on the smallest distance from all variables to the linear slope. Linear regression determines the slope that best predicts one variable (y) from the other variable (x). Linear regression was used in paper IV.

Logistic regression is used in paper IV. Logistic regression is used if one of the variables is a nominal variable.

Spearman's rho is a non-parametric[23] correlation test which describes how well two ranked variables correlate with each other. Spearman's rho is used in paper II and III.

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[21] Geometric mean is the average of the entire variables sum.

[22] Nominal variables are also named categorical variables and are variables with labels, in contrast to numerical variables or quantitative variables which are measuring variables on a numerical scale.

[23] Non-parametric refer to data sets that do not follow Gaussian distribution.

ANOVA compares the mean of several unmatched groups; however, in the case of two groups a student's t-test is equivalent. In paper I, one-way ANOVA was used.

Dunnett's test is comparing each group's mean to the mean of the control group with an adjustment for multiple comparisons, without comparing all the groups with each other. Dunnett's test is used in paper I.

Statistical *significance* is reported as a p-value. The p-value does not give the answer to the hypothesis; it is just an indication if the observed difference would give the same result in an exact identical setup. Throughout the papers, differences with a p-value of <0.05 are considered significant, thus the probability of an outcome with no difference between measured data sets are less than 5%, if the experiment is performed with an identical setup.

Summary paper 1-4

- Lyso-PC is biologically active in two different concentration ranges, one nano-molar and one micro-molar concentration range.
- In vitro stimulation of monocytes with lyso-PC stimulates monocytes to adopt a pro-inflammatory phenotype, characterized by increase expression of pro-inflammatory surface markers and release of pro-inflammatory cytokines.
- The lyso-PC level is elevated in plaque extracts from symptomatic patients compared with plaque extracts from asymptomatic patients.
- CCR7 staining is more abundant in plaque from symptomatic patients, compared with plaque from asymptomatic patients. The CCR7 staining is co-localized with staining of CD68 and associated with the lyso-PC content of the plaque.
- Lyso-PC levels correlate with plaque Lp-PLA_2 mass and with pro-inflammatory cytokines in the plaque.
- LDL modified in the presence of an Lp-PLA_2 inhibitor reduced the release of MCP-1 by mononuclear leukocytes, compared with stimulation with LDL modified in the absence of Lp-PLA_2 inhibitor.
- Elevated $CD14^{++}CD16^{-}$ monocytes predicts cardiovascular events.
- $CD16^{+}$ monocytes are inversed associated to carotid IMT at baseline.
- CCR5 expression on $CD14^{+}CD16^{++}$ monocytes is inversed associated to carotid IMT.

Result and discussion

Initiation of the plaque

In the beginning of plaque development, small amounts of LDL particles will be trapped in the *tunica intima*. In the intima, the LDL particles will start to oxidize and give rise to inflammatory mediators, such as lyso-PC. However, in this early stage of atherosclerosis, the concentration of any mediator will probably not be abundant; rather the mediators will only be found in small quantities.

Activation of endothelial cells

In order for the monocytes to enter the artery wall, the endothelial cells need to become activated and up-regulate adhesion molecules that monocytes can bind to. Lyso-PC could be involved in the activation of endothelial cells in early stages of atherosclerosis as nano-molar concentrations induce MIP-2 release from an endothelial cell line named bEnd.3 (paper I). The mouse cytokine MIP-2 or the human equivalent IL-8 has been shown to be up-regulated in endothelial cells by lyso-PC treatment in a recent publication [153].

Recruitment of monocytes

However, it is not enough that the endothelial cell layer is activated; monocytes need to be actively recruited into the artery wall. Nano-molar concentration of lyso-PC functions as a chemo-attractant for migrating monocytes (THP-1 cells) in an *in vitro* transmigration assay (paper I). This is implicating that lyso-PC could be involved in the recruitment of monocytes into the plaque at early stages of atherosclerosis. Also, when stimulating mononuclear leukocytes with lyso-PC overnight, $CD14^+$ monocytes up-regulated surface expression of CCR2, which is the receptor for MCP-1 (paper II). CCR2 has been proven to be important for monocyte migration into the plaque in mice models [154, 155].

Activation of mononuclear leukocytes

Newly immigrated immune cells, are immediately exposed to an inflammatory microenvironment in the plaque. To mimic this situation with lyso-PC in focus, mononuclear leukocytes were stimulated *in vitro* overnight with lyso-PC. Lyso-PC stimulation induced release of pro-inflammatory cytokines (paper II). In addition, the cells increased their expression of CCR2 and decreased their expression of CD163 (paper II). CD163 is a ScR which has been proposed to be a marker for alternatively

activated macrophages [156, 157]. To further establish the role of oxLDL derived lyso-PC, LDL was minimally modified in the presence or absence of the Lp-PLA_2 specific inhibitor SB-435495. The induced release of MCP-1 by mononuclear leukocytes after mmLDL stimulation was inhibited by 40% when mmLDL was modified in the presence of SB-435495 (paper III).

Activation of pro-inflammatory macrophages

In the early stages of plaque development, the inflammation is reversible. However, as the plaque development proceeds, the milieu in the plaque will become more and more pro-inflammatory. In addition to MIP-2 release from endothelial cells, lyso-PC induced transcription and release of MIP-2 in RAW264.7 cells, peritoneal macrophages, and bone marrow derived macrophages (paper I). Not only did lyso-PC induce pro-inflammatory cytokine production and release by differentiated macrophages, lyso-PC did also stimulate differentiation of monocytes into classically activated macrophages (paper II). These macrophages displayed an increase in CCR7 and HLA-DR, and a decrease of CD163. Classically activated macrophages have been shown to have an up-regulation of CCR7 [158, 159], and HLA-DR [156, 157].

Plaque progression

In the early stages of plaque progression, there is still egression of cells. However, as the plaque continues to progress, egression appears to be impaired [116]. Most probably the cause of the impaired egression is multifactorial but lyso-PC could be a part of the explanation.

Entrapment of cells in the plaque

In addition to the chemo-attractant effect at nano-molar concentrations of lyso-PC on monocytes, there is also a chemo-attractant effect at micro-molar concentrations of lyso-PC (paper I). Lyso-PC also induced strong chemoattractants, such as RANTES and MCP-1, by mononuclear leukocytes (paper II). Thus, lyso-PC has both a direct and an indirect effect on retaining the cells within the plaque.

Necrotic core formation and cytotoxicity of lyso-PC

Another contributing factor to plaque progression is lyso-PC cytotoxicity. Micro-molar concentrations of lyso-PC are cytotoxic to cells (paper I). Both nano-molar and micro-molar concentration ranges induced MIP-2 release; however, at nano-molar concentrations no cytotoxicity was detected. It could be that lyso-PC at different concentrations has different biological effects. At high micro-molar concentrations, the MIP-2 release could be a result from a cytotoxic stress mechanism which may contribute to cell death and have an impact on necrotic core formation.

Lyso-PC and atherosclerosis in humans

As shown in *in vitro* experiments, lyso-PC can induce and sustain inflammation. To confirm that the results found *in vitro* also could have an impact *in vivo*, plaque lyso-PC levels, Lp-PLA_2 mass, plaque cytokines and plaque phenotype was evaluated in patients with symptoms or without symptoms. The level of lyso-PC was increased in plaque from symptomatic patients compared with plaque from asymptomatic patients (paper II).

Lyso-PC and macrophages in vulnerable plaques

Levels of lyso-PC in plaque homogenate was correlated to immuno-staining of CCR7 and of CD68[24] confirming an impact of lyso-PC on plaque phenotype (paper II). Immune staining of CCR7 and CD68 were co-localized and increased in plaque from symptomatic patients compared with asymptomatic patients (paper II). There was no difference in staining of CD163 between the two patient groups (paper II). Plaque homogenates from patients with symptoms displayed an up-regulation of the pro-inflammatory cytokines IL-6, RANTES, TNFα, and MIP-1β compared with homogenates from asymptomatic patients (paper II) and there was a correlation between lyso-PC and IL-6, RANTES, TNFα, MCP-1, IL-1β, and MIP-1β (paper III). In addition, there was an inverse correlation of the anti-inflammatory cytokine IL-10, and eotaxin with lyso-PC (paper III). IL-10 has been shown to be up-regulated in specimens from patients with un-stable angina compared with specimens from patients with stable angina [160], suggesting that IL-10 could be up-regulated as a response to the inflammation [161].

Lyso-PC and correlation with Lp-PLA_2 mass and plasma level of LDL

Lp-PLA_2 mass was increased in plaque homogenate from symptomatic patients compared to asymptomatic patients and correlated to lyso-PC level (paper II and III). Although, there was a correlation between Lp-PLA_2 mass in plaque homogenate and Lp-PLA_2 mass in plasma, Lp-PLA_2 mass in plasma did not correlate with lyso-PC levels in plaque homogenate (paper III). Plasma levels of C reactive protein, total cholesterol, triglycerides, or HDL did not correlate to lyso-PC, but there was a correlation between LDL levels in plasma and lyso-PC levels in the plaque homogenates (paper III).

Lyso-PC and plaque characteristics

Lyso-PC and Lp-PLA_2 was inversely correlated with smooth muscle cell stained area and calcium content (paper III). Both high calcium and smooth muscle cell content

[24] The ScR CD68 binds oxLDL and is used as a pan-macrophage marker.

has been associated with a more stable plaque phenotype [162]. Lyso-PC also correlated with macrophages and with lipids, both measured with staining of plaque sections (paper III). A high macrophage and lipid content has been associated with unstable plaques [163].

Monocyte in blood from patients with symptoms

In symptomatic patients, expression of CX3CR1, CD127, and CCR7 on $CD14^{++}CD16^{-}$ monocyte displayed a strong trend towards a reduction, compared with $CD14^{++}CD16^{-}$ monocytes from asymptomatic patients (paper II). Interestingly, the population of $CD14^{++}CD16^{-}$ monocytes also predicts cardiovascular events (paper IV). Both $CD16^{+}$ monocytes and CCR5 expression on $CD14^{+}CD16^{++}$ monocytes were inversely associated with intima-media thickness (IMT) (paper IV).

General discussion

Lyso-PC mechanisms

Lyso-PC has been proposed to be a ligand for the orphan receptor G2A [164], but this proposition has been questioned [165]. It should be noted, however, that two of the first articles implicating a role for lyso-PC as a ligand for G2A has later been retracted [166, 167]. Two other G-protein couple receptors GPR4 and OGR1, has also been proposed to mediate lyso-PC signaling [168-170].

The first report implicating a role of lyso-PC in sepsis came 2003 and showed that lyso-PC was reduced in sepsis patients compared to healthy blood donors [171]. Moreover, lyso-PC reduction was more prominent in non-survivors compared to survivors [171]. When lyso-PC was administered therapeutically in experimental sepsis the survival rates of cecal ligation and punctured mice and lipopolysaccharide (LPS[25]) injected mice, increased [172]. Despite the pro-inflammatory effect of lyso-PC itself, lyso-PC has been shown to inhibit the pro-inflammatory properties of LPS [172, 173]. The inhibition of LPS signaling, measured by TNF-α release was shown to be reversed by the use of a G2A blocking antibody [172, 173]. Interestingly, LPC acyltransferase[26] (LPCAT) has been shown to abolish the effect of G2A antibody blockade of lyso-PC inhibition of LPS signaling and inhibit the translocation of

[25] LPS is found on gram-negative bacteria and evokes immune responses through binding to TLR4. LPS is a large molecule consisting of both a lipid part (lipid A) and a polysaccharide.

[26] LPCAT use lyso-PC as a substrate to generate CoA and PC.

TLR4 to lipid rafts[27] [173]. The inhibitory effect of lyso-PC on LPS signaling was also observed in an experiment performed by us, in bone marrow derived macrophages (unpublished observations).

Lyso-PC could mediate biological effects through other mechanisms than a direct interaction with a receptor, such as through palmitoylation of proteins. It has been known for a long time that α-subunits of G-protein couple receptors are palmitoylated [174, 175]. Also, the palmitoylation of α-subunit of G-protein coupled receptors is required for the localization of the α-subunit to caveolae[28] [176, 177], and it has been proposed that palmitoylation of the α-subunits could activate G-protein coupled receptors or promote protein-protein interaction between G-protein coupled receptors and caveolin[29] [174, 177]. Interestingly, lyso-PC has been shown to enhance and stabilize surface expression of G2A in a murine T-cell line, but the direct mechanism is not known [178]. The palmitoylation of the α-subunit of G-protein coupled receptors is reversible and mediated by an acylprotein thioesterase (APT1[30]), an enzyme that use lyso-PC as a substrate [179, 180]. APT1 is also released by the murine macrophage cell line RAW264.7 upon LPS stimulation [180]. Lipid A, a part of the LPS molecule has also been shown to be palmitoylated, making it less active [181]. However, there is no formal proof for palmitoylation playing a role in the studies included in this thesis.

Lyso-PC has also been shown to reduce the permeability of the cell membrane and change to the ionic redistribution across both plasma and mitochondrial membrane [182, 183]. In this way, lyso-PC could without the need for a specific receptor change the behavior of the cell by disrupting membrane integrity. Also, the induced effect seen could be a result of cytotoxicity, where the concentration of lyso-PC is above the critical micelle concentration [183]. The cytotoxicity of lyso-PC is proposed to be a result of lyso-PC induced oxidative stress [183]. Lyso-PC induced cytotoxicity has been suggested to occur above 30-50 µmol/l [184]. However, different cell populations have different sensitivities for lyso-PC induced cytotoxicity (unpublished

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[27] Lipid rafts are microdomains on the cell surface, that organizes signaling molecules and are involved in signaling and trafficking over the cell membrane.

[28] Other proteins have also been shown to require palmityolation for the localization to caveolae including Src kinases and eNOS.

[29] The protein caveolin is concentrated in invaginations on the cell surface, so called caveolae and has been proposed to have a role in concentrating signaling molecules to caveolae, and modulate signaling through receptors.

[30] APT1 was previously known as lysophospholipase 1 (LPL1).

observations). Lyso-PC can effectively be bound to serum albumin, lipoprotein particles, and erythrocytes, presumably making lyso-PC biologically inactive [185]. The percentage of serum added to cell cultures dose-dependently inhibited both MIP-2 release and LDH release from RAW264.7 cells stimulated with lyso-PC (paper I). Interestingly, in normolipidemic patients with hypoalbuminemia lyso-PC is increased in VLDL and LDL [186], suggesting that albumin is a better acceptor of lyso-PC than lipoproteins.

It could also be that lyso-PC itself is biologically inactive. Lyso-PC can be converted into lysophosphatidic acid by the enzyme autotaxin [187]. Lysophosphatidic acid has been ascribed many biological functions and has been proposed to exert its effect through at least 4 different G-protein coupled receptors [188]. Lyso-PC has also been proposed to work as a "come and eat me" signal released from apoptotic cells [189]. The induced migration of THP-1 cells towards apoptotic cells was blocked by addition of lyso-PC, but not by addition of lysophosphatidic acid to counteract migration to the THP-1 cells [189]. Moreover, the chemoattractant agent released from apoptotic cells was proposed to be lyso-PC [189].

Activation of protein kinase C (PKC) is another mechanism by which lyso-PC has benn suggested to act [190, 191]. PKC is activated by diacylglycerol or by Ca^{2+} influx. Even though lyso-PC has been suggested to work as a PKC activator, lyso-PC has also been shown to mediate a PKC independent impairment of endothelium-dependent relaxation [192, 193]. Thus, PKC cannot be the only explanation to lyso-PC mediated effects. The activation of PKC by lyso-PC could also be a result of the Ca^{2+} influx through L-type Ca^{2+} channels, shown to be opened by lyso-PC [194]. Ca^{2+} influx has also been shown after lyso-PC treatment of cultured human endothelial cells [195]. However, the Ca^{2+} influx could also be a result of apoptosis [196], caused by cytotoxic concentrations of lyso-PC. Due to differences in cell populations and serum levels, and the lack of cytotoxicity assays, it is difficult to interpret the role of lyso-PC cytotoxicity in these studies.

Monocytes and macrophages

Monocyte counts are correlated to increased risk of cardiovascular disease [197, 198]. However, how different monocyte subpopulations are associated to cardiovascular risk has not yet been highlighted. In paper IV, there was an inverse correlation between the baseline measurements of intima-media thickness and both of the $CD16^+$ monocyte subpopulations[31]. These findings are supported by studies in high risk patients [81, 82]. In mice, more has been done to characterize the monocyte

[31] The $CD16^+$ monocyte population consists of non-classical monocytes ($CD14^+CD16^+$) and intermediate monocytes ($CD14^{++}CD16^+$).

subpopulations and how the monocyte subpopulations relate to macrophage subpopulations in humans. The human $CD16^{+}$ monocyte subpopulation has been shown to be analogous to the mouse Ly-$6C^{low}$ monocyte subpopulation, with low or absent levels of CCR2, high levels of CCR5, and they are morphologically distinct as these cells are smaller and less granular than the Ly-$6C^{high}$ monocytes [199]. The Ly-$6C^{low}$ monocytes have been proposed to have a role in normal tissue homeostasis, as resident macrophage precursors [7], and have also been shown to differentiate into alternatively activated macrophages when challenged with *L. monocytogenes* infection [200]. The increased $CD16^{+}$ monocyte population that was inversely correlated to IMT could potentially contribute to an increased number of resident macrophages within the plaque, with an increased clearance of the plaque components and down-regulation of the inflammation as a consequence. In a hypercholesterolemic mouse model, Ly-$6C^{low}$ monocytes were partially dependent on CCR5 to enter plaques [201]. Ly-$6C^{high}$ monocytes that almost completely lack surface expression of CCR5 have also been showed to be dependent on CCR5 to enter the plaques [201]. This could however been explained by a migration promoting role of Ly-$6C^{low}$ monocytes on the Ly-$6C^{high}$ monocytes rather than a direct effect of the Ly-$6C^{high}$ monocytes. Interestingly, CCR5 expression on $CD14^{++}CD16^{+}$ monocytes was inversely correlated to IMT at baseline (paper IV). Low CCR5 expression on the $CD14^{++}CD16^{+}$ monocytes could be due to migration of the $CCR5^{+}$ monocytes into the plaques, moving the $CCR5^{+}$ monocyte population from the blood into the plaques. Even though this monocyte population have been ascribed a role in tissue homeostasis, in this way they could promote other monocyte subpopulations to follow into the lesions, such as the $CD14^{++}CD16^{-}$ monocytes subpopulation, thereby promoting inflammation.

$CD14^{++}CD16^{-}$ monocytes subpopulation was shown to predict cardiovascular events and correlated with reduced event-free survival (paper IV). The $CD14^{++}CD16^{-}$ monocyte subpopulation is analogous to the mouse Ly-$6C^{high}$ monocyte subpopulation, which has high levels of CCR2 and low levels of CCR5 [199]. Ly-$6C^{high}$ monocytes have been proposed an inflammatory role due to their ability to populate sites of infection [202], and they have been suggested to be the precursors of plaque macrophages [203]. Disruption of the CCR2 signaling pathway in *Apoe*$^{-/-}$ mice reduces atherosclerosis [154, 155]. When mononuclear leukocytes were stimulated with lyso-PC 16:0 overnight, CCR2 levels increased on the $CD14^{+}$ monocyte population (paper II). Indicating that lyso-PC could activate monocytes and promote their migration into the plaque. When $CD14^{+}$ monocytes were differentiated with growth factors into macrophages, addition of lyso-PC did not induce CCR2 expression on the fully differentiated macrophages (paper II), which is in accordance with the finding that mature macrophages lose their expression of CCR2 [204]. Instead, differentiation of macrophages in the presence of lyso-PC increased the percentage of $CCR7^{high}$ cells, induced expression of HLA-DR, and decreased the

expression of CD163. This macrophage phenotype obtained by lyso-PC stimulation resembles the phenotype of classically activated macrophages [156-159]. Classically activated macrophages are also named pro-inflammatory or M1 macrophages due to their ability to produce type 1 cytokines. To further establish that lyso-PC has a pro-inflammatory role, cytokines were measured after overnight stimulation of mononuclear leukocytes with lyso-PC (paper II). Lyso-PC induced release of IL-6, RANTES, TNFα, and IL-1β, indicating that lyso-PC has a pro-inflammatory role. In paper I, there was also transcription and release of MIP-2 after lyso-PC stimulation on several different cell types. MIP-2 has been shown to be strongly up-regulated in M1 differentiated macrophages compared to M2 differentiated macrophages [205]. Taken together, this is implicating a role for lyso-PC in promoting a macrophage phenotype that is comparable to the phenotype of classically activated macrophages.

With lyso-PC 16:0 in focus, the difference between carotid plaques from patients with and without symptoms was measured. Lyso-PC levels were elevated in plaque from symptomatic patients compared with asymptomatic patients. The elevated levels of lyso-PC were accompanied with an elevation of Lp-PLA_2 levels (paper III). An elevation in Lp-PLA_2 levels would be expected because carotid plaques from symptomatic patients also have increased lipid levels and more macrophages[32] than carotid plaques from asymptomatic patients (paper II). Lp-PLA_2 elevation has been shown to correlate with carotid plaque symptoms in patients [206, 207]. When lyso-PC levels were compared with cytokine levels and to plaque macrophage phenotype, the comparison revealed similarities to the *in vitro* findings. The cytokine profile in plaque homogenates from patients with symptoms resembled the cytokine profile obtained from mononuclear leukocytes stimulated with lyso-PC (paper II). In sections of human carotid plaques from symptomatic patients, the percentage of CCR7 staining was increased compared with asymptomatic patients, and the CCR7 staining correlated to the lyso-PC level (paper II). Lyso-PC was also correlated to the percentage of CD68 staining (paper II). CD68 and CCR7 staining were co-localized which could mean that the main cell population displaying CCR7 are macrophages. However, there were no indications that lyso-PC did affect the alternatively activated subpopulation of macrophages since there were no difference in CD163 in plaques from symptomatic and asymptomatic patients (paper II). Altogether, this is indicating a role for lyso-PC in altering the balance of classically and alternatively activated macrophages, favoring classically activated macrophages.

[32] As mentioned in the section "lipid oxidation", two of the major sources of Lp-PLA_2 in plaque is LDL trapped in the intima and Lp-PLA_2 synthesized by macrophages.

Until a few years ago, macrophages in the plaque were just macrophages. Today macrophages in the plaque are divided into at least two accepted functionally distinct subpopulations and there will most probably be more macrophage subpopulations discovered [205]. Markers for both the classically and the alternatively activated macrophage subpopulations have been found together in human plaques indicating that both are present at the same time [208]. The macrophages have been regarded as pro-atherogenic, but macrophages are probably not only detrimental for the plaque. Macrophages have an important role in the clearance of both lipids and dead cells in the plaque and the alternatively activated macrophages could induce a down-regulation of the inflammation in the plaque [209]. This suggests that the "right" macrophage subpopulation could be beneficial for the plaque and have a role in stabilizing the plaque.

Treatments

Until recently, it was thought that it was favorable to inhibit inflammation to reduce atherosclerosis. Today, evidence is gathering of the negative role of the non-steroidal anti-inflammatory drugs (NSAID) on cardiovascular risk [210, 211]. NSAIDs have an overall anti-inflammatory effect on cells involved in inflammation and could potentially inhibit both the wanted and the non-wanted immune response simultaneously.

The treatments available today can only reduce the risk of cardiovascular events by approximately 40-50% [212]. If we selectively could inhibit the classically activated macrophage subpopulation or find the mechanism to induce a shift to a more alternatively activated phenotype, the risk of cardiovascular event could potentially be reduced even more. The data in this thesis propose that inhibition of lyso-PC production, as a complement to the treatments available today, could result in a more favorable situation in the lesions, with further reduction of the cardiovascular risk as a consequence.

Interpretation of the data

What you see is not always what you get. A decrease in one factor could be a reflection of an increase in another factor. Also, the decrease of surface markers on monocytes could be due to a decreased release of those monocytes from bone marrow or an enhanced migration of that particular monocyte population into tissue. This is reflected in the results, which can sometimes be difficult to interpret.

We show that lyso-PC is inducing up-regulation of CCR2 on monocytes and that there is a lyso-PC associated increase of CCR7 in human carotid plaques (paper II). Lyso-PC also induces increased release of chemokines, such as RANTES, and MCP-1 (paper II). Most of the chemokine receptors have more than one ligand; some of the receptors have up to seven known ligands i.e. CCR1 and CCR3 [213]. The ligands also possess the ability to bind to more than one receptor [213]. The effect from one

ligand acting on several different receptors may not be consistent, neither is the effect of several different ligands on one receptor [213, 214]. This implies that the combined effect of ligands and receptors is complex and may vary from time to time. As an example, CCR5 expression on $CD14^{+}CD16^{++}$ is negatively associated with IMT (paper IV). CCR5 is the receptor for RANTES, MIP-1α, and MIP-1β among others, suggesting a pro-atherosclerotic effect of CCR5. However, eotaxin also binds to CCR5 and pretreating human monocytes with eotaxin, has been shown to induce internalization of CCR5 and render the monocytes less responsive towards RANTES and MIP-1β [215]. Moreover, eotaxin which was inversely correlated with lyso-PC was also reduced in plaque homogenate from symptomatic patients compared to asymptomatic patients (paper III). It could be speculated that even though CCR5 seem to be pro-atherogenic, a chemokine such as eotaxin could abrogate CCR5 signaling.

The forgotten

The dendritic cells are worth to mention. For a long time, dendritic cells were thought of as $CD11c^{+}$ cells that had as main function to activate cells from the adaptive immunity. Today, our knowledge is increased and unfortunately the division of monocytes, macrophages, dendritic precursor cells, and dendritic cells are more confusing than ever. Monocytes can be precursor cells for macrophages and for dendritic cells, dendritic precursor cells can develop into macrophages and dendritic cells [9, 216]. This "cross-differentiation" seems to occur mainly during inflammation. Also, the marker CD11c that was earlier thought of as marker exclusively expressed on dendritic cells is now found to be expressed on 90 % of all human monocytes [9]. In the plaque, CD68 has been used as a marker for macrophages; however, it is now shown that CD68 is also expressed on dendritic cells in the plaque [217]. Maybe in the context of inflammation, the dendritic cell or the macrophage should not be considered as a separate cell type with unique capacity or destiny but rather as subsets of mononuclear phagocytes [218].

In this thesis, the effect of lyso-PC on the adaptive immune system was not evaluated, neither was the effect of different macrophage subpopulations on the adaptive immune system. Although the adaptive immune system was not specifically studied in lyso-PC induced cytokine release from mononuclear leukocytes, the cytokine release cannot be attributed only to the monocytes. Mononuclear leukocytes are composed of both monocytes and lymphocytes, and the cytokine release after lyso-PC treatment of mononuclear leukocytes is probably a multicellular response reflecting the interplay between the adaptive and the innate immune system. Moreover, the HLA-DR up-regulation on the lyso-PC stimulated macrophages together with the release of type 1 cytokines could potentially induce a pro-inflammatory shift in the adaptive immune system. It would be interesting to address these effects of lyso-PC on the adaptive immune system.

Also, the effect of other Lp-PLA_2-produced mediators was not evaluated. As an example, oxidized non-esterified fatty acids have been ascribed a role that probably will contribute to the inflammation [68]. However, the effect of other Lp-PLA_2-produced mediators than lyso-PC will not diminish the contribution of lyso-PC.

In the end

Why has the body a system with enzymes like Lp-PLA_2 which results in production of mediators such as lyso-PC? It is not that lyso-PC is in any way evil; lyso-PC is just slightly misunderstood. In the early stages of atherosclerosis, Lp-PLA_2 and the production of lyso-PC has probably an important role in the induction of LDL clearance in the intima and in mediating danger signals to the immune system. But thereafter, something goes wrong and atherosclerosis escalates. The plaque reaches a point of no return and the body is incapable to cope with the inflammation.

In addition to the traditional risk factors, danger signals, such as elevated levels of $CD14^{++}$ cells, which can predict cardiovascular events, could be important to distinguish not only individuals with a high risk to develop cardiovascular disease but also individuals with high risk of developing rupture-prone plaques. In these individuals with a high risk of developing rupture-prone plaques, an immuno-modulating treatment pinpointed on pro-atherosclerotic mechanisms, such as lyso-PC production, would be desirable.

Populärvetenskaplig sammanfattning

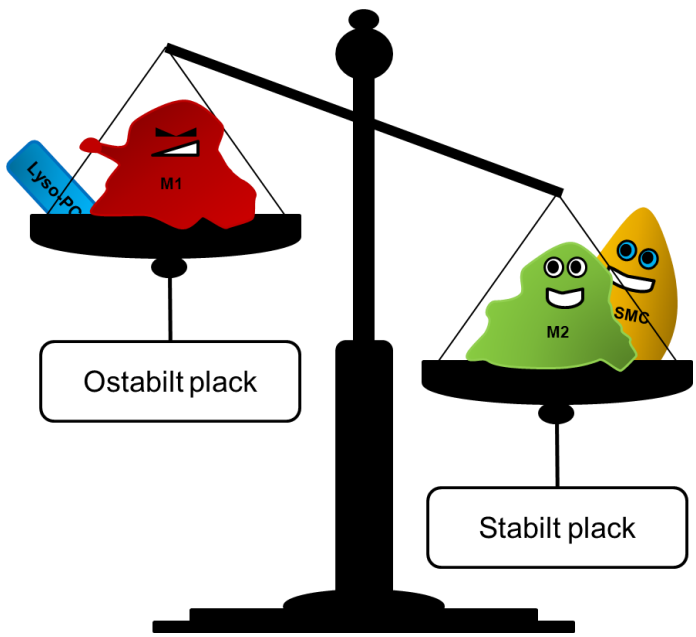
Kardiovaskulär sjukdom är ett samlingsnamn för sjukdomar som involverar hjärtat och/eller kärlen. De är orsaken till 41 % av alla dödsfall i Sverige, och 12 % av alla svenskar lider av följderna av dessa sjukdomar. Ateroskleros, som är en kardiovaskulär sjukdom, kan upptäckas redan hos barn. Då ser man ateroskleros som en förändring i kärlväggen (plack) och i detta stadie är sjukdomen inte farlig. Men med åren blir placken större och det finns risk att de går sönder. Ett plack som går sönder kan ge blodproppar som kan hindra blodflödet och ge skador på vävnader, som i värsta fall leder till döden. Ateroskleros drabbar främst de stora och medelstora artärerna. Artärer är de blodkärl som leder bort från hjärtat och transporterar syre- och näringsrikt blod till kroppens olika delar. Kärlets vägg består av flera lager där endotelceller är det innersta lagret. Endotelcellerna ligger som tegelstenar mot blodet och deras roll är att hålla blodet och blodets komponenter i kärlet. Utanför endotelcellerna finns ett lager med protein och glatta muskelceller, detta lager håller ihop kärlet och gör det elastiskt. Längst ut finns ett lager med kollagen som binder fast kärlet till vävnad runt kärlet. I vissa områden, t.ex. där det finns förgreningar i kärlet, kan det bildas skador. Skadorna gör att endotelcellerna inte kan hålla ihop lika bra och släpper in delar av blodet till den del av kärlväggen som innehåller proteiner och glatta muskelceller. Hos personer med höga halter av fett i blodet, kan fetterna sätta sig i dessa skadade områden. Om cellerna runt om i kärlet inte lyckas ta bort fettet, börjar det att härskna (oxidera). Fettet förändras och ett enzym ($Lp\text{-}PLA_2$) försöker då att bryta ner det oxiderade fettet och det bildas lyso-PC. Lyso-PC är en liten molekyl som fungerar som en signal för fara.

När det är fara i kroppen reagerar den med att skicka dit celler från immunförsvaret, immunceller, som kan ta hand om faran. I blodet färdas hela tiden immunceller som kan hjälpa kroppen om det uppkommer fara. En av de första immuncellerna som reagerar vid fara är monocyter. När monocyterna lämnar blodet och går in i vävnaden antar de en ny skepnad och blir makrofager. Makrofagernas uppgift är att döda bakterier och virus (om dessa är faran) men också att identifiera faran och berätta för andra celler vilken typ av fara som har tagit sig in i kroppen. De är också väldigt duktiga på att städa upp skräp i vävnaden, som döda bakterier och delar av vävnaden som gått sönder. Makrofagerna städar genom att äta upp skräpet. När kroppen reagerar på skada och fara bildas det en inflammation. Om kroppen inte kan ta bort faran fortsätter inflammationen och den blir då kronisk, detta är vad som händer i placket. Plack kan delas in i två typer. En typ som är stabil och en typ som är ostabil

och som har lätt för att spricka. Ett stabilt plack kännetecknas av att det finns många glatta muskelceller (SMC) och att makrofagerna är av en snäll typ (M2). Ett ostabilt plack har lite glatta muskelceller och har en typ av makrofager som kan beskrivas som arga (M1). (Se bild!)

Under min tid som doktorand har jag undersökt hur lyso-PC påverkar immunceller och hur det påverkar vilken typ av plack som bildas. Jag har visat att lyso-PC gör att monocyter gärna tar sig från blodet och ut i kärlväggen. Där bidrar lyso-PC till att man får den arga typen av makrofager, och mycket Lp-PLA_2 och lyso-PC i placket gör att man får ett ostabilt plack. Jag har också undersökt hur olika typer av monocyter kan påvisa om man senare i livet får ett plack som är ostabilt. Där kunde jag se att i blodet hos personer som riskerar att få ostabila plack, finns fler av den typ av monocyt som man tror lättare kan bli en arg makrofag.

Idag kan man ta olika mediciner som gör att risken för att få ett ostabilt plack minskar med 40 %. Dessa mediciner är vad vi kallar systemiska, d.v.s. de påverkar hela kroppen och inte enbart de celler, enzym eller de molekyler som är farliga. När mediciner påverkar oss systemiskt kan de också vara dåliga för vissa andra processer i kroppen som vi inte vill ska påverkas, därför behövs det att vi utvecklar mediciner som är mer specifika. Idag testar man en medicin som stänger av Lp-PLA_2 som gör lyso-PC, och man har sett i djurförsök att placken blir mer stabila. Jag tror att i framtiden kommer vi ha fler av dessa specifika mediciner som kan skydda fler från att drabbas av kardiovaskulära sjukdomar.



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Abbreviations

ABC	ATP-binding cassette transporter
$ApoB^{100}$	Apolipoprotein B-100
ATP	Adenosine triphosphate
BMI	Body mass index
CCR	C-C chemokine receptor
CCR	C-C chemokine receptor
CD	Cluster of differentiation
cDNA	Complementary DNA
CPIP	Carotid plaque imaging project
DAMP	Damage-associated molecular pattern
ELISA	Enzyme-linked immunosorbent assay
eNOS	Endothelial nitric oxide synthase
FMO	Fluorescent minus one
FSC	Forward scatter
HDL	High-density lipoprotein
HLA-DR	Human leukocyte antigen-DR
HMG-CoA	3-hydroxy-3-methylglutaryl-coenzyme A
IFN	Interferon
IL	Interleukin
IMT	Intima-media thickness
LDH	Lactate dehydrogenase
LDL	Low-density lipoprotein
Lp-PLA_2	Lipoprotein-associated PLA_2
LPS	Lipopolysaccharide

Lyso-PC	Lyso-phosphatidylcholine
MCP	Monocyte chemotactic protein
MDA	Malondialdehyde
MDC study	Malmö diet and cancer study
MHC	Major histocompatibility complex
MIP	Macrophage inflammatory protein
mmLDL	Minimally modified LDL
mRNA	Messenger RNA
NADH	Nicotinamide adenine dinucleotide
NSAID	Nonsteroidal anti-inflammatory drug
oxLDL	Oxidized LDL
oxNEFA	Oxidized non-esterified fatty acid
oxPL	Oxidized phospholipid
PAF	Platelet-activating factor
PAF-HA	PAF acetylhydrolase
PAMP	Pathogen-associated molecular pattern
PDGF	Platelet-derived growth factor
PKC	Protein kinase C
PLA_2	Phospholipase A_2
q-PCR	Real time quantitative polymerase chain reaction
RANTES	Regulated upon Activation, Normal T-cell Expressed, and Secreted
ScR	Scavenger receptor
sdLDL	Small dense LDL
$sPLA_2$	Soluble PLA_2
SSC	Side scatter
TGF	Transforming growth factor
TIA	Transient ischemic attack
TLR	Toll-like receptor
VLDL	Very low-density lipoprotein

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