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**Inflammation-induced atherogenesis,
liver alterations, and
cardiovascular outcome**

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Academic dissertation

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LIST OF ORIGINAL PUBLICATIONS

This thesis is based on the following original publications which are referred to in the text by their Roman numerals.

- I Tuomainen AM, Jauhiainen M, Kovanen PT, Metso J, Paju S, Pussinen PJ. *Aggregatibacter actinomycetemcomitans* induces MMP-9 expression and proatherogenic lipoprotein profile in apoE-deficient mice. *Microbial Pathogenesis* 2008;44:111-117.
- II Tuomainen AM, Hyvärinen K, Jäkälä P, Mervaala E, Leinonen M, Saikku P, Kovanen PT, Jauhiainen M, Pussinen PJ. Proatherogenic microbes promote foam cell formation and endothelial dysfunction in apoE-deficient mice. Submitted.
- III Hyvärinen K, Tuomainen AM, Laitinen S, Bykov IL, Törmäkangas L, Lindros K, Käkälä R, Alfthan G, Salminen I, Jauhiainen M, Kovanen PT, Leinonen M, Saikku P, Pussinen PJ. Chlamydial and periodontal pathogens induce hepatic inflammation and fatty acid imbalance in apolipoprotein E –deficient mice. *Infect Immun* 2009;77:3442-3449.
- IV Tuomainen AM, Nyssönen K, Laukkanen JA, Tervahartiala T, Tuomainen T-P, Salonen JT, Sorsa T, Pussinen PJ. Serum matrix metalloproteinase-8 concentrations are associated with cardiovascular outcome in men. *Arterioscler Thromb Vasc Biol* 2007;27:2722-2728.

In addition some unpublished data are presented.

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ABBREVIATIONS

AAA	abdominal aortic aneurysm
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ac-LDL	acetylated LDL
ACS	acute coronary syndrome
ADMA	asymmetric dimethylarginine
ALT	alanine aminotransferase
AMI	acute myocardial infarction
APC	antigen presenting cell
apo	apolipoprotein
ARA	arachidonic acid
APR	acute-phase response
AST	aspartate aminotransferase
BM	basement membrane
BMI	body mass index
CAD	coronary artery disease
CD36	cluster of differentiation 36
CD40L	CD40 ligand
CE	cholesteryl ester
CETP	cholesteryl ester transfer protein
CHD	coronary heart disease
CMV	cytomegalovirus
CRP	C-reactive protein
CVD	cardiovascular diseases
DGLA	dihomo γ -linoleic acid
DNA	deoxyribonucleic acid
ECM	extracellular matrix
EB	elementary body
eNOS	endothelial nitric oxide synthase
FAS	fatty acid synthase
FFA	free fatty acid
GLA	γ -linoleic acid
HDL	high density lipoprotein
HL	hepatic lipase
HSP	heat shock protein
ICAM	intercellular adhesion molecule
IDL	intermediate density lipoprotein
IFN- γ	interferon-gamma
IL	interleukin
IMT	intima-media thickness
i.v.	intravenously
LBP	lipopolysaccharide binding protein
LCAT	lecithin:cholesteryl acyltransferase
LDL	low density lipoprotein
LDLr	low density lipoprotein receptor
LPL	lipoprotein lipase
LPS	lipopolysaccharide

LXR	liver X receptor
MCP-1	monocyte chemoattractant protein-1
M-CSF	macrophage colony-stimulating factor
MetS	metabolic syndrome
MHC	major histocompatibility complex
MI	myocardial infarction
MMP	matrix metalloproteinase
MPO	myeloperoxidase
mRNA	messenger ribonucleic acid
MT-MMP	membrane-type matrix metalloproteinase
MTPP	microsomal triglyceride transfer protein
MUFA	mono-unsaturated fatty acid
NAFLD	nonalcoholic fatty liver disease
NASH	nonalcoholic steatohepatitis
NF- κ B	nuclear factor-kappa B
NO	nitric oxide
ox-LDL	oxidized LDL
PAMP	pathogen-associated molecular pattern
PCR	polymerase chain reaction
PECAM-1	platelet endothelial cell adhesion molecule-1
PLTP	phospholipid transfer protein
PON-1	paraoxonase-1
PUFA	poly-unsaturated fatty acid
RB	reticulate body
RCT	reverse cholesterol transport
RNA	ribonucleic acid
ROS	reactive oxygen species
SAA	serum amyloid A
SFA	saturated fatty acid
SMC	smooth muscle cell
sPLA ₂ -IIa	group IIa secretory phospholipase A ₂
SR-A	scavenger receptor type A
SR-BI	scavenger receptor type BI
SREBP	sterol regulatory element-binding protein
TG	triglyceride
TGF- β	transforming growth factor β
Th cell	T helper cell
TIMP	tissue inhibitor of metalloproteinases
TLR	toll-like receptor
TNF- α	tumor necrosis factor- α
VCAM	vascular cell adhesion molecule
VLDL	very low density lipoprotein

ABSTRACT

Cardiovascular diseases, which presently are considered inflammatory diseases, affect millions of people worldwide. Chronic infections may contribute to the systemic inflammation suggested to increase the risk for cardiovascular diseases. Such chronic infections are periodontitis and *Chlamydia pneumoniae* infection. They are highly prevalent as approximately 10% of adult population and 30% of people over 50 years old are affected by severe periodontitis and 70-80% of elderly people are seropositive for *C. pneumoniae*.

Our general aim was to investigate the role of infection and inflammation in atherosclerosis both in animal and human studies. We aimed to determine how the two pathogens alter the atherosclerosis-associated parameters, and how they affect the liver inflammation and lipid composition. Furthermore, we evaluated the association between matrix metalloproteinase-8 (MMP-8), a proteinase playing a major role in inflammation, and the future cardiovascular diseases (CVD) events in a population-based cohort.

For the animal experiments, we used atherosclerosis-susceptible apolipoprotein E deficient (apoE^{-/-}) mice. They were kept in germ free conditions and fed with a normal chow diet. The bacteria were administered either intravenously (*A. actinomycetemcomitans*) or intranasally (*C. pneumoniae*). Several factors were determined from serum as well as from aortic and hepatic tissues. We also determined how cholesterol efflux, a major event in the removal of excess cholesterol from the tissues, and endothelial function were affected by these pathogens. In the human study, serum MMP-8 and its tissue inhibitor (TIMP-1) concentrations were measured and their associations during the follow-up time of 10 years with CVD events were determined.

An infection with *A. actinomycetemcomitans* increased concentrations of inflammatory mediators, MMP production, and cholesterol deposit in macrophages, decreased lipoprotein particle size, and induced liver inflammation. *C. pneumoniae* infection also elicited an inflammatory response and endothelial dysfunction, as well as induced liver inflammation, microvesicular appearance and altered fatty acid profile. In the population-based cohort, men with increased serum MMP-8 concentration together with subclinical atherosclerosis (carotid

artery intima media thickness > 1mm) had a three-fold increased risk for CVD death during the follow-up.

The results show that infections with *A. actinomycetemcomitans* and *C. pneumoniae* induce proatherogenic changes, as well as affect the liver. These data therefore support the concept that common infections have systemic effects and could be considered as cardiovascular risk factors. Furthermore, our data indicate that, as an independent predictor of fatal CVD event, serum MMP-8 could have a clinical significance in diagnosing cardiovascular diseases.

1. REVIEW OF THE LITERATURE

1.1. Atherosclerosis and inflammation

1.1.1. Introduction

Cardiovascular diseases, with atherosclerosis as the underlying cause, are in Europe the most common cause of death in men under 65 years, and the second most common cause in women, and its prevalence is increasing in the developing countries. The concept of atherosclerosis being just a lipid storage disease leading to blockage of blood flow has predominated the thinking of the disease. During the past decades the crucial involvement of inflammation has been recognized. It is now evident that inflammation affects all stages of atherosclerosis: initiation, progression and eventually complications. Atherosclerosis has the same basic mechanisms as other inflammatory diseases, i.e. immune system secretes factors affecting the epithelial and mesenchymal cells of the organ in question. In the case of atherosclerosis, it is the vascular wall with its vascular endothelial cells and smooth muscle cells (SMC), respectively. This process leads to e.g. leukocyte recruitment, extracellular matrix (ECM) remodelling and cellular proliferation.

The vascular wall is composed of three layers. Intima is lined with a monolayer of endothelial cells facing the lumen and internal elastic lamina on the peripheral side. Intima is mainly composed of ECM components, collagen and proteoglycans. The middle layer, media, contains SMCs, and the third layer, adventitia, is formed of connective tissues as well as fibroblasts and SMCs. (1) Atherosclerosis starts with accumulating lipids and immune cells into the intima, thus forming fatty streaks. These fatty streaks contain lipid-laden macrophages, so called foam cells. Fatty streaks may further progress into atherosclerotic lesions containing foam cells, dead cells, and lipid droplets. These components compose the core of the lesion which is surrounded by a fibrous cap containing collagen and SMCs. (1-3) Lesions first grow towards adventitia, after which they expand to lumen. They may grow, as mononuclear cells from the blood stream enter the lesion, and also due to cell proliferation, ECM production and further accumulation of lipids. Eventually, after years or even decades, the advanced lesion, atheroma, may rupture forming thrombus thus either partially or totally

blocking the blood flow to the tissue. Lesions mostly develop at the area where the blood flow is disturbed, such as arterial branching points, rather than in regions with laminar flow. (1,2,4)

1.1.2. Overview of lipid metabolism

The transport of cholesterol is mediated by different lipoprotein particles that are composed of lipids and apolipoproteins. The core of the particle is hydrophobic containing esterified cholesterol and triglycerides (TGs), and it is surrounded by a hydrophilic layer of phospholipids, free cholesterol and apolipoproteins. There are five major classes of lipoproteins: chylomicrons, very low density lipoproteins (VLDL), intermediate density lipoproteins (IDL), low density lipoproteins (LDL), and high density lipoproteins (HDL).

Cholesterol comes from two sources: exogenously from the diet and endogenously by synthesis. Cholesterol received from the diet is absorbed in the intestine and packed into TG-rich lipoproteins called chylomicrons (Figure 1). Chylomicrons are largely composed of TGs and to a lesser extent of cholesterol, and the main apolipoprotein is apoB-48. Lipoprotein lipase (LPL) bound to endothelium hydrolyzes TGs of chylomicrons in the circulation and the resulting chylomicron remnants are delivered to the liver. Hepatic cholesterol, which is mainly endogenously synthesized by the hepatocytes, is together with TGs packed as VLDL with apoB-100 being the major apolipoprotein (Figure 1). TGs of VLDL are further hydrolyzed by LPL resulting in the formation of VLDL remnants, IDL, which are either taken up by the liver or converted to LDL by hepatic lipase (HL). Approximately two thirds of plasma cholesterol is carried by LDL with apoB-100 being the predominating apolipoprotein. LDL functions as the source of cholesterol for steroidogenesis and cellular membranes. It is taken up by the liver or peripheral cells via apoB-100 recognizing LDL-receptor (LDLr). The liver clears LDL from the circulation, but excess amounts of LDL results in an elevated plasma cholesterol concentration. Unlike cells in the liver and the steroidogenic tissues, most cells are unable to metabolize cholesterol. Instead they regulate their LDL receptors in a way to control cholesterol content for membrane integrity and other functions. However, some cell types, such as macrophages can take up excess cholesterol via scavenger receptors resulting in the accumulation of cholesterol in e.g. arterial wall eventually leading to atherosclerosis. (5-8)

Cholesterol from peripheral tissues is transported to the liver for excretion by HDL in a process called reverse cholesterol transport (RCT). One third of the plasma cholesterol is carried by HDL, and approx. 70% of its total protein content is composed of apoA-I (6). Liver synthesizes and secretes apoA-I which interacts with ATP-binding cassette transporter A1 (ABCA1) on the surface of peripheral cells, particularly macrophages, and the liver (Figure 1) (9). The naive lipid-free apoA-I is lipidated with free cholesterol and phospholipids forming partially lipidated discoidal HDL. HDL disk matures into spherical HDL via esterification of cholesterol by lecithin:cholesterol acyltransferase (LCAT). HDL particles can accept cholesterol from the macrophages also via ATP-binding cassette transporter G1 (ABCG1) after lipidation of apoA-I by ABCA1 (10). From HDL, the formed cholesteryl esters (CEs) are either transported to the liver by hepatic scavenger receptor BI (SR-BI) for bile secretion, or to other lipoproteins containing apoB, such as LDL through cholesteryl ester transfer protein (CETP) which exchanges CEs for TGs (11). Phospholipids are transferred by phospholipid transfer protein (PLTP) from TG-rich lipoproteins to HDL during lipolysis by LPL. PLTP also mediates fusion of small HDL particles into larger ones. Mature HDL particles are further modified by PLTP by removing phospholipids to maturing HDL and by HL that hydrolyzes TGs and phospholipids (Figure 1). Therefore, the actions of CETP, PLTP and HL remodel mature HDL particles releasing lipid-poor apoA-I for the next round of lipidation. (6,12,13)

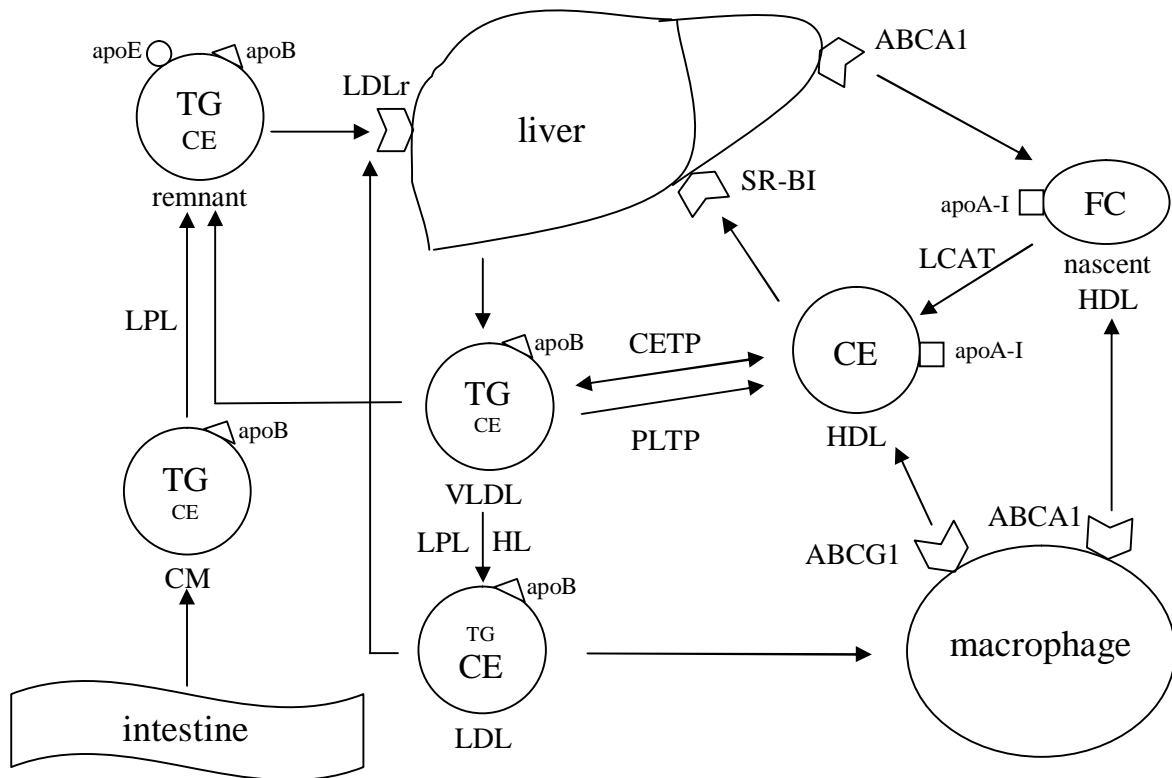


Figure 1. Lipoprotein metabolism. Modified from (14).

1.1.3. Inflammatory mechanisms in atherogenesis

1.1.3.1. Innate and adaptive immunity

Both innate and adaptive immunity are involved in atherosclerosis. The innate immune response is a rapid, non-specific response to pathogen-associated molecular patterns (PAMPs), such as lipopolysaccharide (LPS) and modified LDL. Macrophages, the main effector cells of innate immunity, express pattern recognition receptors, such as scavenger receptors and Toll-like receptors (TLRs). The ligation of scavenger receptors leads to endocytosis. TLR ligation activates e.g. nuclear factor-kappa B (NF- κ B) pathway. These result in production of reactive oxygen species (ROS) as well as release of cytokines and adhesion molecules, which further affect the inflammatory status of the tissue. The innate immunity affects also the adaptive immunity as activated macrophages express major histocompatibility complex (MHC) class II antigens that are needed for antigen-dependent activation of T-cells. (15-18)

The other defence system, adaptive immunity, is slower and more specifically recognizing molecular structures and involving T- and B-cells. Antigen presenting cells (APCs), such as dendritic cells and macrophages present foreign antigens to T-cells, which thereafter produce a response specifically to that antigen. The response may be a cytotoxic T-cell attack against the cell bearing the antigen, stimulation of B-cell antibody production and induction of local inflammatory response. T-cells can differentiate into either T helper 1 (Th1) or T helper 2 (Th2) cells, Th1 cells being more prominent than Th2 in atherosclerotic lesion. (19-21) Th1 cells secrete cytokines, of which interferon-gamma (IFN- γ) is the most common and mediates the crosstalk between adaptive and innate immunity by stimulating macrophage production of ROS and pro-inflammatory cytokines such as tumor necrosis factor- α (TNF- α) and interleukin-1 (IL-1) (21,22). IFN- γ also inhibits collagen production and cholesterol efflux, induces production of adhesion molecules, enhances T-cell and macrophage recruitment, lipid uptake by macrophages, activation of APCs, and proliferation of SMCs (2,20). Th2 cells, on the other hand, secrete cytokines such as IL-10, which also functions as an anti-inflammatory cytokine, that stimulate maturation of antibody-producing B-cells (21,23). They also may recruit and activate another inflammation-related cell type, namely mast cells (24). Another differentiated T-cell form, regulatory T-cell, suppresses the functions of other immune cells and secretes transforming growth factor β (TGF- β), an atheroprotective cytokine whose targets in atherosclerosis are mainly T-cells, but also endothelial cells, dendritic cells, SMCs and macrophages. It also stabilizes lesions via increased collagen synthesis and MMP inhibitor production. T-cell activation is not necessary for the initiation but for the early progression of atherosclerosis. (2,22)

1.1.3.2. Effects of inflammation on the initiation and progression of atherosclerosis

The initial step in the formation of atherosclerosis is adhesion of monocytes to the vessel wall (Figure 2). Normal, healthy vessels can resist the adhesion. However, in situations that trigger atherogenesis, such as lipid accumulation into the intima due to diet and modification of LDL, smoking, hypertension, insulin resistance, microbes, or pro-inflammatory cytokines (such as TNF- α and IL-1 β), endothelial cell expression of adhesion molecules results in the monocyte attachment (1,25). The adhesion molecules include vascular cell adhesion molecule-1 (VCAM-1), intercellular adhesion molecule-1 (ICAM-1), and P- and E-selectins. After the endothelium binding, a chemokine called monocyte chemoattractant protein-1 (MCP-1) secreted by endothelial cells, SMCs, and macrophages, recruits monocytes which penetrate

the endothelium and enter the intima causing accumulation of monocytes. Once in the intima, macrophage colony-stimulating factor (M-CSF) induces monocyte maturation into macrophage and macrophage proliferation. Both MCP-1 and M-CSF are overexpressed in atherosclerotic lesions (26-29). Macrophages start to increase expression of TLRs and scavenger receptors. Scavenger receptors internalize modified LDL resulting in cholesterol ester accumulation in the cytoplasm and converting the cells into so-called foam cells, lipid laden macrophages associated with the early atherosclerotic lesion formation. Signals through TLRs lead to macrophage activation and mediate the release of cytokines, proteases, and ROS. (1,15,30) This cascade leads to the formation of fatty streaks that are asymptomatic and found already in young people, but do not necessarily progress to more advanced atherosclerotic lesions (31).

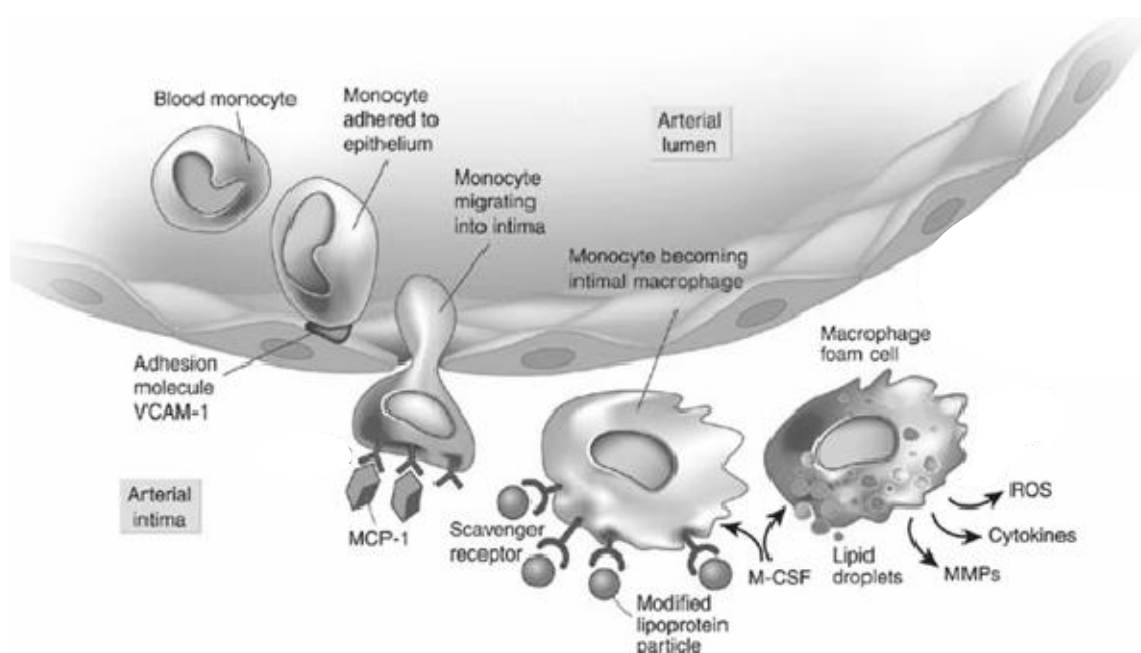


Figure 2. Monocyte recruitment and foam cell formation. Reproduced with permission from (32).

In the intima, macrophages secrete e.g. ROS and cytokines that sustain the inflammatory status in the vessel wall. The inflammatory status is further supported by pro-inflammatory cytokines produced by T-cells. Pro-inflammatory signals also induce production of inflammatory mediators by endothelial and SMCs. (16) During the progression of atherosclerosis, CD40 ligand (CD40L) participates in the immune response. T-cell CD40L ligates with B-cell CD40 leading to reduced B-cell apoptosis, enhanced B-cell amplification, and activated T-cell pro-inflammatory cytokine production (33). CD40 and CD40L are

expressed also in macrophages, vascular endothelial cells and SMCs (34,35). CD40L regulates the expression of adhesion molecules, cytokines, and chemokines, as well as induces MMP and tissue factor production, all involved in the atherogenesis (1,36-41). T-cells are always present in atherosclerotic lesions, especially CD4+ cells that recognize MHC class II bound antigens (19). Human atherosclerotic lesions have been shown to contain CD4+ cells reactive to *Chlamydia pneumoniae* proteins, oxidised (ox)-LDL, and *Porphyromonas gingivalis* heat-shock protein (HSP) 60 (42-44) .

The main structure protecting the atherosclerotic lesion is the fibrous cap. It can resist the function of most proteases, but some MMPs are capable of initiating the cleavage of the triple helical molecule structure of collagen, the main component of the cap. Activated macrophages, SMCs, and endothelial cells present in atherosclerotic lesions express several MMPs that can degrade the collagen structure of the cap (45-48). Several inflammatory mediators, such as an acute phase protein serum amyloid A (SAA), TNF- α , and IL-1, are able to induce the expression of MMPs (46,47,49). In addition to collagen degradation, also the collagen synthesis is reduced due to pro-inflammatory cytokines. T-cells affect both the collagen degradation by activating macrophages and the reduced collagen synthesis by e.g. IFN- γ -mediated inhibition of collagen messenger ribonucleic acid (mRNA) expression (22,50). Once the cap has ruptured, the thrombotic core of the lesion is exposed and released to the lumen enabling the interaction between macrophage-derived tissue factor and blood resulting in coagulation and thrombus (51). Inflammatory processes are thus present in all stages of atherosclerosis: initiation, progression, and the actual CVD event.

1.1.4. Mouse as an atherosclerosis model

Basic research of atherosclerosis is mostly based on animal models as studying it in humans is difficult due to its complexity, chronicity and impossibility to characterize lesions sequentially. A number of animal models have been used, such as pigs and rabbits, but the most widely used are mice. Mice have the advantage of being easy, fast and economical to breed, easy and economical to maintain, their environment and diet can be controlled, the genetic information on many inbred strains is available, and they are the most widely used mammals in experiments involving genetic manipulations. Also the size of the animal permits studies on e.g. pharmacological agents to be carried out, as novel drugs are not synthesized in

large quantities and are therefore sufficient only for studies in small animals. Due to the size and easy maintenance, group numbers and animal numbers in the groups can be large, which is necessary to achieve reliable data. This is particularly important in atherosclerosis studies, as the lesion sizes vary greatly due to large natural variance. Other drawbacks associated with the small body size are the low amount of blood available, small tissue sizes for analyzation, and restricted surgical possibilities. (52,53)

Probably the main disadvantage of mice in atherosclerosis research is that they do not develop atherosclerosis spontaneously. Mice are HDL animals carrying most of their cholesterol in HDL, whereas humans have approximately 70% of their plasma cholesterol in LDL. In inbred mice strains, the capability to develop diet-induced atherosclerosis varies greatly, C57Bl/6 strain being the most susceptible. Yet, feeding of a long term lipid-enriched diet causes lesion growth only in the aortic root in C57Bl/6 mice. This has been overcome by generating genetically modified strains where larger lesions form either spontaneously or after proper diet. These include apoE^{-/-} mice with C57Bl as a background strain. However, no mouse strain has been observed to develop plaque rupture in spite of the presence of intraplaque hemorrhage in mature lesions. (53,54)

There are some differences between humans and mice from the atherosclerotic point of view. As mentioned, wild type mice are HDL animals, mostly due to the lack of CETP. This makes them generally resistant to atherosclerosis. Genetically modified mouse strain expressing CETP has an increased cholesterol content of VLDL and LDL. In humans, liver produces only apoB-100, whereas in mice both apoB-100 and its shorter form apoB-48 are produced; in humans apoB-48 is produced in the intestine. The structure of apoA-II differs, as it is dimeric in humans and monomeric in mice. Mice also lack lipoprotein(a). Humans produce HL in the liver and it is bound to hepatocytes and hepatic endothelial surfaces, whereas it is free in the circulation in the mice. Atherosclerotic plaques are most frequently present in murine aorta and not in coronary arteries like in humans, and they also develop much faster in mice compared to those in humans. The plaques do not necessarily cause symptoms seen in humans, such as myocardial infarction (MI), cardiac dysfunction and occlusive coronary artery disease. (52) However, a double knockout model (SR-BI^{-/-}/apoE^{-/-}) has developed a complete coronary artery occlusion resulting in MI, reduced heart function, cardiac conductance defects and early death (55). In addition, there are many immunological

differences between mice and humans (56) and also the endotoxin dose must be many times higher in mice than in men to induce corresponding effects (57).

The most widely used mouse model in atherosclerotic research is apolipoprotein E deficient (apoE^{-/-}) mice that was generated in 1992 by inactivating the gene in mouse embryonic stem cells via homologous recombination (58). ApoE is present in lipoprotein particles being a major component of VLDL, and serves also as a ligand to LDL-receptor thereby enabling the uptake of apoE-containing particles by the liver. As a result of this, the characteristics of apoE^{-/-}-mice include increased total cholesterol (approx. 8-11 mmol/l compared to 2 mmol/l in C57Bl/6) and triglyceride concentrations and decreased HDL cholesterol concentration on a regular chow diet. These mice show very early, at the age of 5-6 weeks, monocyte adhesion and migration. Fatty streaks with foam cells and migrating SMCs start to appear in 6-10 week-old mice. Lesions grow rapidly to advanced lesions with a necrotic core surrounded by proliferating SMCs and ECM. The plaques appear along the entire arterial tree; more pronounced plaques appearing in the aortic arch and in the aortic sinus. In older mice, lesions have calcified foci. At the age of one and half years, the occlusion of arteries can be as high as 90%. If these mice are given diet enriched with fat, the progression of lesions accelerates significantly with occlusions appearing in less than one year. As previously mentioned, the genetic background influences the susceptibility to develop atherosclerosis, and therefore the most widely used background in apoE^{-/-} mouse is the C57Bl/6. The lipoprotein profile of apoE^{-/-} mice differs from that of humans, as they carry most of their plasma cholesterol in VLDL. However, their suitability as an atherosclerotic model is well justified as their lesion progression and cell types as well as oxidized lipoproteins are similar to those found in humans. (52-54,59,60)

1.2. Infection in atherosclerosis

1.2.1. Background

Atherosclerosis is an inflammatory disease, and one of the culprits may be infections caused by pathogens. As early as in the 1970s germ-free chickens infected with avian herpesvirus were found to produce human atherosclerosis-like disease (61). Since then, several pathogens have been associated with CVD such as *Chlamydia pneumoniae*, periodontal pathogens, cytomegalovirus (CMV), and *Helicobacter pylori*.

Pathogens may be linked to development and progression of atherosclerosis by several mechanisms. Most investigations are based on seroepidemiological studies, but it has also been shown, that infectious agents may reside in the vessel wall e.g. by invading endothelial cells, like in case of *C. pneumoniae* and periodontal pathogens (62-66). CMV infection can cause SMC replication probably by inhibiting tumour suppressor gene p53, as its product inhibits cell cycle progression, or by enhancing growth factor secretion or receptor expression (67,68). Both CMV and *C. pneumoniae* are able to inhibit apoptosis of infected cells and CMV has also been shown to increase SMC migration; both factors affecting the mass growth of lesions (67,69,70). Infections and LPS also cause accumulation of lipids in macrophages due to down-regulation of SR-BI and ABCA1, whereas scavenger receptor type A (SR-A) is up-regulated in inflammation (71-77). Infections result in a release of cytokines, chemokines, and cellular adhesion molecules thus making the endothelium more susceptible to monocyte adhesion (78,79).

One pathogen is, however, unlikely to solely cause development and/or progression of atherosclerosis. Several seroepidemiological studies have shown that multiple infections, so called infectious burden, increases the risk for cardiovascular diseases. There is a correlation between the number of pathogens, both viruses and bacteria, patients are exposed to and the risk for coronary artery disease (CAD), elevated C-reactive protein (CRP) concentrations, extent of atherosclerosis, and cardiovascular death (80-82).

One mechanism, which may contribute to atherosclerotic lesion development due to infection, is molecular mimicry, where pathogen contains a structure homologous to that in a host

protein resulting in production of autoantibodies. Especially pathogen-derived HSPs, HSP60 or GroEL, have been shown to cross-react with human HSP60, as in case of *Helicobacter pylori*, CMV, *Escherichia coli*, *C. pneumoniae* and periodontal pathogen *Porphyromonas gingivalis* (83-86). High antibody titers against HSPs are linked to atherosclerosis, and endothelial cells ubiquitously express HSP60 in response to blood pressure, hypercholesterolemia, cytokines, low-shear stress and LPS (87,88). Molecular mimicry also exists between epitopes of oxidized LDL (ox-LDL) and *Streptococcus pneumoniae* (89). As all bacteria have HSPs, and they are highly conserved, it is likely that many other pathogens may also be involved in these inflammatory reactions. Altogether, pathogens may induce an inflammatory status in the arterial wall leading to atherosclerosis via mechanisms described in section 1.1.3.

1.2.2. Periodontitis

1.2.2.1. Introduction

Periodontitis is a chronic bacterial infection affecting gingiva and tooth-supporting tissues. It is an infection of multiple, mostly gram-negative anaerobic bacterial species, such as *Aggregatibacter* (previously *Actinobacillus*) *actinomycetemcomitans*, *Porphyromonas gingivalis*, *Prevotella intermedia*, and *Tannerella forsythia* (previously *Tannerella forsythensis*) (90). Over 700 different bacterial species present in dental pockets have been cultured (91,92). Periodontal infection initiates as plaque at gingival margin gradually transform to dental calculus and finally invades and degrades the connective tissue and bone support (Figure 3). The loss of tooth support is mainly due to host defence, such as secretion of proteases, and results in formation of deepened periodontal pockets. If the disease is not treated, it will lead to loss of teeth. Periodontitis is a common disease worldwide; approximately 10% of adult population and 30% of people over 50 years of age suffer from severe periodontitis (93,94). In Finland, 64% of adults have signs of periodontitis, and the severe forms affect 21% of the adult population (95).

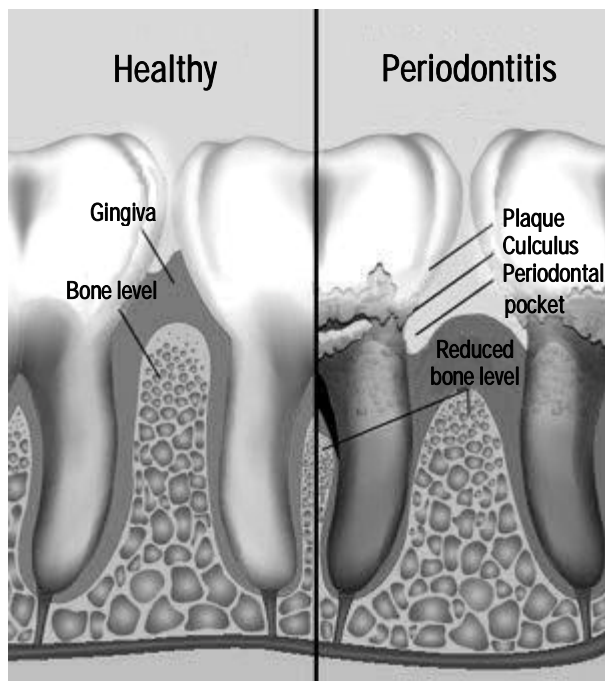


Figure 3. Periodontitis. The host response to periodontal pathogens and dental calculus leads to reduction of connective tissue and bone support resulting in deepened periodontal pockets and attachment loss.

1.2.2.2. Periodontitis and cardiovascular diseases

Periodontitis has been proposed to be one of the infections associated with atherogenesis, and it was initially suggested by Mattila et al in 1989 (96). Periodontal pathogens and their LPS continuously have a direct access to the systemic circulation via inflamed periodontal pockets as a result of e.g. tooth-brushing and eating, thereby inducing systemic inflammation and LPS responses as described in section 1.2.5. This theory is supported by the fact that periodontal pathogens and their deoxyribonucleic acid (DNA) have been found in human atherosclerotic lesions (97-99) and that they are able to invade aortic endothelial and smooth muscle cells *in vitro* (65,66,100) thus likely affecting the endothelial integrity. Proinflammatory cytokines released from inflamed periodontal pockets may induce inflammatory effects not only locally but also systemically (101), and for example CRP, a risk factor for CVD, is elevated in periodontal patients compared to periodontally healthy subjects (102). Periodontitis and the resulting inflammatory cytokines can also induce endothelial dysfunction and platelet aggregation, that are also CVD risk factors (101,103,104). Several studies have shown that periodontitis or exposure to periodontal pathogens increase the risk for CVD as well as cause increase in intima-media thickness (IMT), which can be considered as a marker of subclinical

atherosclerosis (105-108). Periodontitis also affects lipid metabolism. It associates with increased LDL and TG levels and decreased HDL concentration as well as macrophage activation and foam cell formation (73,109-114). After periodontal treatment, many atherosclerosis-related markers improve, including CRP and other inflammatory mediators, cholesterol concentrations, and LDL particle sizes (111,112,115,116) further implying the systemic and proatherogenic effects of periodontitis.

1.2.2.3. *Aggregatibacter actinomycetemcomitans* and atherosclerosis

One major periodontal pathogen is *Aggregatibacter actinomycetemcomitans*, a facultative, gram-negative anaerobe. It is especially prevalent in juvenile periodontitis as approximately 90% of these patients carry *A. actinomycetemcomitans* compared to 50% of adult patients, but it may also be present in healthy individuals in lower proportions (117,118). Several human and animal studies have implied the possible role for *A. actinomycetemcomitans* in CVD. In serological studies antibodies to *A. actinomycetemcomitans* associate with prevalent CVD (119-121) as well as increased risk for future CVD events such as stroke, coronary heart disease (CHD), and MI (108,122,123). *A. actinomycetemcomitans* has been detected by immunohistochemistry (124) and by polymerase chain reaction (PCR) (97,125-129) from vascular tissues of CVD patients. Furthermore, viable bacteria have been isolated from human atherosclerotic plaques (98). According to Spahr et al. the periodontal pathogen burden, assessed by DNA-DNA hybridization, and especially the presence of *A. actinomycetemcomitans*, is associated with increased risk of CHD (130). A recent study concerning MI and antibodies against periodontal bacteria also showed the significance of presence of several bacterial strains instead of just one (131). Despite of the clinical significance of *A. actinomycetemcomitans*, there are only a few animal studies concerning the association of the pathogen and CVD. These studies, however, show that *A. actinomycetemcomitans* e.g. induces mast cell activation in the aortic sinus (132).

1.2.2.4. *Porphyromonas gingivalis* and atherosclerosis

P. gingivalis is a gram-negative obligate anaerobe, and is especially associated with adult aggressive and chronic forms of periodontitis. Its atherogenic effects have been studied extensively. *P. gingivalis* serology not only shows association between exposure to *P. gingivalis* and prevalent CVD (119,133,134), but also the risk for future CVD events, such as

stroke and MI (108,122,123,135,136). The presence of *P. gingivalis* in atherosclerotic plaques has been verified immunohistochemically and by PCR (97,124,127,128,137). Also, like *A. actinomycetemcomitans*, viable invasive *P. gingivalis* bacteria have been identified in human atherosclerotic plaques (98). In animal studies, *P. gingivalis* given to apoE^{-/-} mice either orally, anally (due to coprophagic nature of mice), or intravenously (i.v.) results in accelerated atherosclerosis, occurrence of bacterial ribosomal DNA in the aortas, livers and hearts, and increased expression of aortic VCAM-1, tissue factor, TLR-2 and TLR-4 (138-140). Atherosclerotic lesion development has also been detected in rabbits and pigs infected with *P. gingivalis* (141,142). The pathogen could, furthermore, be detected in pig arteries by PCR (142). Overall the results indicate that the continuous access of periodontal pathogens to the blood stream may affect the initiation and progression of atherosclerosis.

1.2.3. *Chlamydia pneumoniae*

1.2.3.1. Introduction

The order *Chlamydiales* comprises a large number of bacteria that have a common feature of obligate growth in eukaryotic cells. The order classically contains one family, *Chlamydiaceae*, one genus, *Chlamydia*, and four species, *C. trachomatis*, *C. pneumoniae*, *C. psittaci*, and *C. pecorum* (143). A more recent, and controversial, proposed taxonomy divides the family *Chlamydiaceae* into two genera, *Chlamydia* containing three and *Chlamydophila* six species, and additional three new families would be formed from *Chlamydia*-like bacteria (*Waddliaceae*, *Parachlamydiaceae*, and *Simkaniaceae* (144). In this taxonomy, *Chlamydia pneumoniae* is part of the genus *Chlamydophila*, but the new classification has also been considered unnecessary (145).

C. pneumoniae is a gram-negative bacterium causing respiratory tract infection, and studies concerning prevalence of antibodies to *C. pneumoniae* show that the infection rate is high; approximately 70-80% of elderly people are seropositive, males more often than females, but it is less common in children under five years in industrialized countries (146). The manifestations of the disease are numerous, as it can vary from asymptomatic to life-threatening pneumonia (147). *C. pneumoniae* is transmitted as metabolically inactive particle which differentiates, replicates, and re-differentiates in a host cell. *C. pneumoniae* has two

forms, the elementary body (EB) and the reticulate body (RB). The infectious form, EB, is small (ca. 0.3 μm), round, and metabolically inert. It invades the host cell and is internalized in a vacuole called inclusion. Inside the cell, EB differentiates into the metabolically active, non-infectious RB that is larger (ca. 1 μm) and divides repeatedly ('binary fission'). RBs undergo secondary differentiation back to EBs that are released upon cell lysis and are able to infect the surrounding cells. In chronic state, *Chlamydia* is associated with the host cell staying in a viable but culture-negative form. (148) *C. pneumoniae* infection is suggested to be linked to several diseases, one of them being atherosclerosis.

1.2.3.2. *Chlamydia pneumoniae* and atherosclerosis

The association between *C. pneumoniae* and atherosclerosis was initially proposed by Saikku et al. over twenty years ago, when they discovered elevated antibody response in patients with acute myocardial infarction (AMI) or chronic CHD (149). Since then, the research concerning this association has bloomed, and dozens of seroepidemiological studies have further verified the concept. A stronger evidence of this association has come from the studies showing the presence of *C. pneumoniae* in e.g. atherosclerotic plaques. The first evidence of the presence of *C. pneumoniae* in lesions came from transmission electron microscopy study of atherosclerotic lesions from autopsy tissues (150) that was later confirmed by immunohistochemistry and PCR (62). The viability of *C. pneumoniae* in lesions was shown few years later, as viable bacteria from coronary artery were cultured; the presence of bacteria was demonstrated also by PCR, immunohistochemistry, electron microscopy, and *in situ* hybridization (151). Viable *C. pneumoniae* has also later been detected in atherosclerotic lesions (152). As many as almost 60% of atherosclerotic lesions contain *C. pneumoniae* compared to 3% in normal vessel wall (153). PCR results can vary greatly between studies, but altogether the data clearly indicate the presence of *C. pneumoniae* in atherosclerotic lesions thereby likely contributing to proatherogenic changes. However, not all studies have confirmed this association between *C. pneumoniae* and atherosclerosis (154).

Basically two animal models have been used in *C. pneumoniae* studies concerning atherosclerosis, that is rabbits and mice. New Zealand white rabbits show early atherosclerotic changes such as foam cell formation in the aortic arch (155) and intimal thickening (156,157) after *C. pneumoniae* inoculations. In mouse studies, hyperlipidemic strains such as apoE^{-/-} show more advanced lesions (158-160), as well as in LDLr^{-/-} (161,162), apoE3-Leiden (163),

and wild type (164-166) mice fed a cholesterol-rich diet. In contrast, in normolipidemic mice *C. pneumonia* do infect and induce inflammatory changes in the aorta, but do not show indications of further lesion progression (167,168) indicating the importance of cholesterol in the ability of *C. pneumoniae* to induce proatherogenic changes.

The mechanism by which *C. pneumoniae* contributes to atherosclerosis may be partly explained by its ability to enter vasculature and vascular wall via monocytes and lymphocytes (169). *C. pneumoniae* is able to infect and replicate in endothelial and SMCs, as well as in macrophages (64,170). It also increases endothelial expression of adhesion molecules (79) and enhances attachment of infected monocytes to the endothelium (171). *C. pneumoniae* causes LDL oxidization (172,173) and its LPS induces macrophage foam cell formation (72). All these effects cause inflammatory response of the vasculature and thus enhance atherogenesis.

1.2.4. Lipopolysaccharide

Lipopolysaccharide is the outer membrane structure of gram-negative bacteria, and it is also termed as endotoxin. Gram-negative bacteria are common human pathogens, including *Escherichia coli*, *Salmonella enterica*, *C. pneumoniae*, *P. gingivalis*, and *A. actinomycetemcomitans* which colonize human gastrointestinal, genitourinary, oral cavity, and respiratory tracts and produce endotoxins not only during clear infections but also during chronic inflammatory conditions. The outer membrane of gram negative bacteria is a bilayer composed of mainly LPS containing outer leaflet and inner leaflet rich in phospholipids and proteins. The functions of the outer membrane are to communicate with the environment by harbouring and transporting nutrients, protecting the bacteria from toxic compounds such as antibiotics, or interacting with the host. LPS also causes the physiological effects, such as fever, during infection and it can be released from the bacteria upon multiplication or death and lysis. (174,175)

1.2.4.1. Structure of LPS

LPS is composed of three regions: O-specific side chain, core oligosaccharide, and lipid A, of which O-side chain protrudes outwards and lipid A attaches LPS to the bacterium (Figure 4).

The O-side chain is a heteropolysaccharide consisting of repeating oligosaccharide units, and possesses therefore an enormous variability. Carbohydrate residues in each repeating unit have different antigenic properties; these are called ‘O-factors’. Several O-factors can be present in one repeating unit thus determining the serotype of the bacteria. The core region consists of heterooligosaccharide and is less variable than the O-side chain. The most conserved part of LPS is the lipid A, the bioactive component of LPS causing fever and lethal toxicity. (176) It is also responsible for macrophage activation and most of the endotoxic effects of LPS, which actually appear due to macrophage activation and the following cytokine as well as ROS release. LPS interacts not only with macrophages but also with e.g. B-cells, T-cells, vascular endothelial and SMCs (174,175,177) inducing the production of pro-inflammatory cytokines and adhesion molecules (178,179).

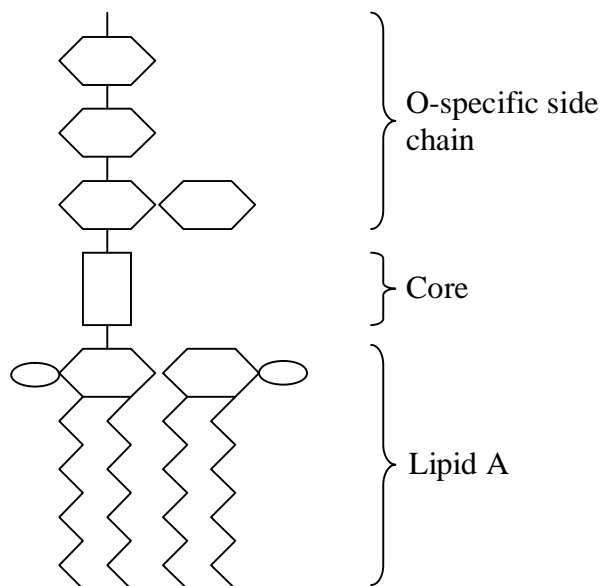


Figure 4. Structure of LPS.

1.2.4.2. Receptors of LPS-mediated signalling

For mediating signals to cells, LPS forms a complex with several proteins, i.e. LPS binding protein (LBP), CD14, TLR-4, and MD-2. Together these proteins initiate the signalling that via activation of kinases results in the activation of NF- κ B. LBP binds LPS and delivers it to CD14 or lipoproteins (180,181). LPS-CD14 complex further reacts with membrane bound TLR-4 and its co-receptor MD-2, which binds both to TLR-4 and LPS (182,183). In periodontal patients the concentration of soluble CD14 is elevated (184) indicating an

increased LPS exposure. TLR-4 is expressed and mediates LPS response in vascular endothelial cells, and it is also present in lipid rich atherosclerotic plaques containing macrophages (185-187). Not only LPS, but also e.g. HSP60, fibronectin, minimally modified LDL, and saturated fatty acids can function as ligands for TLR-4 (188-190). The induction of NF- κ B leads to the activation of e.g. interleukins, adhesion molecules, MCP-1, and MMPs (15). Activation of TLR also blocks liver X receptor (LXR) target gene ABCA1 (191), thus affecting the cholesterol efflux.

1.2.4.3. LPS neutralization and clearance by lipoproteins

LPS is cleared from the circulation by lipoproteins which receive LPS from LBP or PLTP (181,192,193). LPS binds via lipid-A to lipoproteins, predominantly to HDL, but clearly also to LDL and VLDL (177,194). LPS binds rapidly to HDL, and it is further redistributed mainly to LDL by PLTP (194,195). In septic conditions, but also in periodontitis, when HDL concentration is lowered, the binding of LPS is shifting towards VLDL and its overall transfer rate to lipoproteins is accelerated (114,196,197). The main factor in neutralizing the LPS activity is the phospholipid content of the lipoproteins together with apolipoproteins, such as apoE and apoA-I (14,198). Indeed, one function of lipoproteins is to protect the body from lethal dose of LPS; TG-rich lipoproteins transfer LPS further to hepatocytes, where it is deactivated and secreted into bile (199-202).

1.2.5. Infection and lipid metabolism

Infection and inflammation alter lipid and lipoprotein metabolism. During acute phase response (APR), plasma triglyceride and VLDL levels increase whereas HDL concentration decreases. The increased TG concentration is likely to result from increased VLDL either due to increased VLDL production or impaired VLDL clearance. In rodents, low LPS dose enhances VLDL production by enhancing lipolysis of adipose tissue, increasing hepatic FA synthesis, and lowering hepatic FA oxidation, whereas high LPS dose reduces LPL activity resulting in diminished VLDL clearance. (203-205). VLDL clearance is further affected by the infection-related reduction in apoE expression (206).

The composition and structure of lipoproteins are also affected by infection. VLDL and LDL are enriched with sphingolipids making them more likely proatherogenic (207,208). LDL particles become small and dense being thus more easily oxidized (209), having reduced LDLr binding affinity (210), and having capability to penetrate the endothelium and to effectively bind proteoglycans in the intima (211). These changes enhance the accumulation of LDL particles in the arterial wall and foam cell formation.

HDL functions as an anti-atherogenic lipoprotein as it i) mediates RCT, ii) scavenges or breaks down oxidized phospholipids, iii) suppresses the induction of adhesion molecules, iv) reduces ROS expression, v) increases endothelial nitric oxide synthase (eNOS) activity and concentrations, as well as reverses the ox-LDL-mediated decrease in nitric oxide (NO) production, vi) has anti-thrombotic properties, and vii) represses apoptosis of endothelial cells (212,213). In APR, HDL is remodelled converting it from anti-inflammatory to pro-inflammatory lipoprotein. As HDL mediates RCT, the processes involved in modification of HDL or other components related to RCT may thus have adverse effect on cholesterol export from peripheral cells to the liver.

During APR, in HDL particles apoA-I is replaced by SAA resulting in a decrease in apoA-I concentration (214,215). Anti-inflammatory enzymes associated with HDL, such as paraoxonase-1 (PON-1) and platelet-activating factor acetylhydrolase that otherwise inactive oxidized lipids are decreased in acute-phase HDL (216,217). Pro-inflammatory cytokines coordinate the reciprocal hepatic expression of SAA, apoA-I, and PON-1; they increase SAA expression and at the same time decrease apoA-I and PON-1 expression (218). Lipid-free SAA promotes cholesterol efflux not only via ABCA1 like apoA-I, but unlike apoA-I, also in a ABCA1- and energy-independent way (219). There are, however, conflicting results concerning the role of SAA in RCT, as some studies indicate it to increase RCT (220-222), whereas others suggest the opposite (223-225). However, HDL enriched with SAA binds rather macrophages than hepatocytes (226). SAA functions also as a chemoattractant alluring inflammatory cells to the site of inflammation (227,228). It has been suggested that in acute inflammation increased SAA may be beneficial, but deleterious if chronically increased as in type 2 diabetes (229).

During inflammation, apoA-I may be modified by macrophage-derived myeloperoxidase, MPO, leading to decreased ability of HDL to remove cholesterol via ABCA1 (230,231).

Khovidhunkit et al have shown, that endotoxin or cytokines, TNF- α and IL-1, decrease both ABCA1 and ABCG1 mRNA as well as their protein levels in J774 murine macrophages (75) therefore reducing cholesterol efflux. HDL concentration decreases in APR e.g. due to remodelling of HDL by an acute phase protein group IIa secretory phospholipase A2 (sPLA₂-IIa) as it has been shown to increase HDL catabolism (232). It also converts LDL into small, dense and thus more atherogenic particles (233). sPLA₂-IIa has been detected in atherosclerotic lesions (234). However, mice with C57Bl/6 genetic background have a point mutation making the sPLA₂-IIa non-functional (235).

The scavenger receptors of macrophages are involved in the uptake of modified LDL (236). Class A scavenger receptors SR-AI and SR-AII are expressed in macrophages, including foam cells, as well as in aortic endothelial cells and vascular SMCs. They are involved in the uptake of acetylated and ox-LDL as well as recognizing gram-negative bacteria (237-239). Class B receptor CD36 binds e.g. moderately ox-LDL, as well as native lipoproteins (240,241) and its function is required for foam cell formation (242). Another class B receptor, SR-BI is involved in selective cholesterol transfer to and from HDL (11), as previously described in RCT. Both receptor classes are affected by LPS administration, which in experimental research mimicks infection; SR-A expression is upregulated (243), whereas hepatocytic SR-BI expression is decreased (74) (Figure 5). In the RAW 264.7 macrophage cell line LPS stimulation causes a decrease in SR-BI mRNA level resulting in either unchanged or decreased protein levels (73,74,76). APR affects also enzymes related to cholesterol metabolism. It lowers the activities of HL, LPL (244), CETP (245), and LCAT (246), and increases PLTP activity (247). The effects of infection on lipid metabolism are summarised in Figure 5. Due to all these effects infection has on lipid metabolism, the lipid accumulation increases in peripheral tissues and atherosclerosis development progresses.

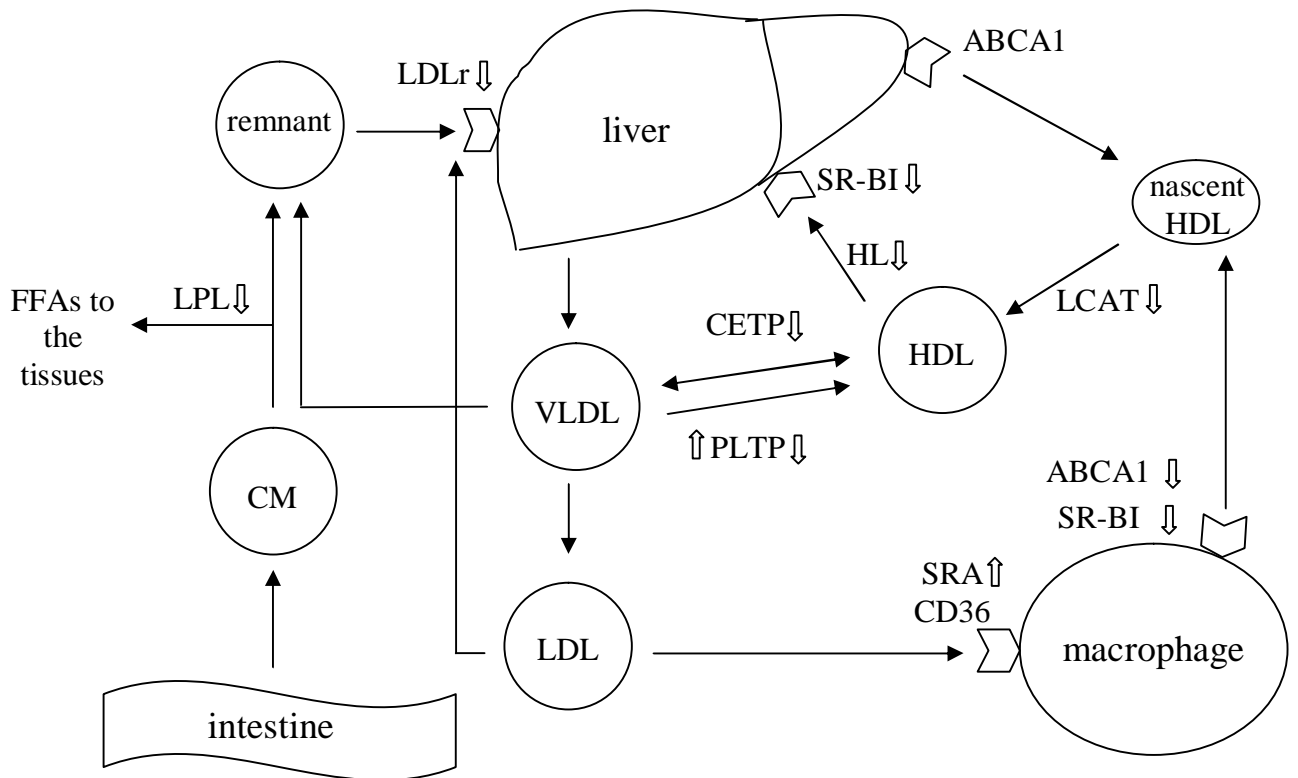


Figure 5. Effects of infection on lipid metabolism. PLTP activity has been shown to decrease in mouse and increase in human. Modified from (14).

1.2.6. Endothelial function

Healthy vascular endothelium has diverse functions. It participates in the vasodilation, and inhibits adhesion and migration of circulating leukocytes, proliferation and migration of SMCs, and adhesion and aggregation of platelets (248). These functions are compromised in endothelial dysfunction that is a marker of atherosclerosis.

One key function of endothelium is vasodilation. The key regulator of vasodilation is NO, which is synthesized from L-arginine by eNOS present in the lipid rafts called caveolae (249). In inflammation LDL is oxidized and this ox-LDL induces translocation of eNOS by taking up caveolae cholesterol via CD36 making stimulation of eNOS impossible (250). This can, however, be prevented by HDL (251) which binds SR-BI in caveolae resulting in activation of eNOS (252); normal caveolae cholesterol levels and eNOS association are thus maintained. This may be disturbed in atherosclerosis as HDL is modified and its concentration decreases

at the same time as ox-LDL concentration increases thus affecting the vasodilation properties of vasculature. Also inflammatory mediators, such as TNF- α , downregulate the activity of eNOS (253), as well as increase the production of ROS which is able to reduce NO activity (254,255). Oxidative stress also increases the concentration of asymmetric dimethylarginine (ADMA), an endogenous eNOS inhibitor, (256). As both native and ox-LDL increase ADMA concentration, lipid accumulation may affect endothelial function via ADMA (257,258).

Another marker of endothelial dysfunction is increased expression of adhesion molecules, which attract inflammatory cells to the site of inflammation. The upregulated molecules include e.g. selectins (P- and E-selectin) and various adhesion molecules, such as ICAM-1, VCAM-1, and platelet endothelial cell adhesion molecule-1 (PECAM-1). E-selectin is associated with the rolling of cells and it is expressed both in acute and chronic endothelium, but is not constitutively expressed under normal conditions (259). It is expressed in endothelial cells upon exposure to inflammatory cytokines, such as TNF- α , and oxidative stress, and it has been found on the surface areas of plaques (260,261). The adhesion is mediated by VCAM-1 and ICAM-1 that are upregulated by TNF- α , disturbed oscillatory flow common in the regions with atherosclerotic plaques, and by exposure to ox-LDL (88,262-264).

Infections affect endothelium in several ways. In fact, it has been shown, that infections, and particularly infection burden, correlate with the severity of endothelial dysfunction (265). Also other studies with e.g. vaccination or endotoxin administration show impaired endothelial function (266). Patients with impaired endothelial function have increased risk for future cardiovascular events (267). Correspondingly, treatment of atherosclerosis, hypercholesterolemia or chronic infections improves endothelial function (268-270).

1.3. Steatosis

1.3.1. Introduction

Liver takes up lipids both as free fatty acids (FFAs) and lipoprotein particles. Some lipoprotein particles are taken up by endocytosis whereas in others the TGs are hydrolyzed by HL producing FFAs that are able to diffuse or be transported to the liver. Liver also has de novo FA synthesis by fatty acid synthase (FAS), whose expression is regulated by sterol regulatory element-binding protein (SREBP)-1c. (271,272) Free fatty acids may further be oxidized by β -oxidation thus producing both energy and ketone bodies. They may also be used as building blocks for phospholipids and mediators (e.g. prostaglandins and leukotrienes) whereas the carbon skeletons can be utilized in glucose and cholesterol production. FFAs may also be converted to TGs for VLDL production, and the amount of exported VLDL depends not only on the availability of TGs, but also on synthesis of protein components. The protein component of VLDL, apoB, is degraded by insulin action, and SREBP-1c inhibits the formation of microsomal triglyceride transfer protein (MTTP), that catalyzes the lipidation of apoB. The excess TGs are stored as lipid droplets in the hepatocytes. (273)

A substantial source of FFAs is a circulation, where FFAs from dietary fat and adipocytes through lipolysis of stored TGs, are available. If serum FFA levels are elevated for a long period, the liver FA intake may be increased. This may be the case in insulin insensitive or resistant state, such as type II diabetes. (274) Steatosis is a liver condition manifested by TG accumulation in the hepatocytes. As previously described, liver has a key role in lipoprotein metabolism. In steatosis, the FA cycle is distorted resulting in fat accumulation into the liver. Steatosis is defined as a nonalcoholic fatty liver disease (NAFLD), when it develops without excess use of alcohol. It is one of the most common liver diseases worldwide, its prevalence being 20-25% in the general population. Increasing with age, NAFLD tends to be more common in males than in females (275,276). Diabetes and obesity are closely associated with NAFLD, as half of diabetics and approximately 75% of obese people have NAFLD (277-279).

1.3.2. Nonalcoholic fatty liver disease

The spectrum of NAFLD extends from simple steatosis to nonalcoholic steatohepatitis, NASH, possibly leading to fibrosis, cirrhosis and hepatocellular carcinoma. The pathophysiology leading to NAFLD, and especially to hepatocellular damage after TG accumulation, is not yet fully understood. A “two hit” theory has been proposed to cause the NAFLD, the first being the accumulation of fat leading to steatosis, and the second being the oxidative stress due to β -oxidation of fatty acids. Oxidative stress produces lipid peroxidation, to which the steatotic liver is believed to be vulnerable to, and results in NASH. It has been estimated, that 15-20% of patients with NAFLD develop NASH (273).

The accumulated FFAs per se can be toxic to cells in non-adipose tissues. In the liver, this lipotoxicity enhances progression of liver injury. Studies have demonstrated that liver injury and hepatic inflammation correlate with hepatocyte apoptosis. (280). FFAs contribute to apoptosis by activating both the intrinsic and the extrinsic apoptotic pathways (281,282). Saturated fatty acids (SFAs) have been shown to induce apoptosis in e.g. hepatocytes (283) and endothelial cells (284), and also monounsaturated fatty acids (MUFAs) are apoptotic, yet to a markedly lesser extent than SFAs (283).

Even though the primary causes of NAFLD are obesity and insulin resistant state, also several other secondary causes may enhance NAFLD formation. Drugs (corticosteroids, synthetic estrogen, tamoxifen, etc.), surgical procedures (gastroplexy, extensive small bowel resection, jejunio-ileal bypass etc.), toxins (toxic mushrooms, petrochemicals, etc.), metabolic disorders (lipodystrophy, dysbetalipoproteinemia, acute fatty liver of pregnancy, etc.), and several other factors (HIV infection, small bowel diverticulosis with bacterial overgrowth, inflammatory bowel diseases, etc.) are associated with NAFLD (285,286).

Fatty liver disease has been linked to many infections, including both periodontitis and *C. pneumoniae*. In healthy Japanese women, periodontitis associated with steatosis is illustrated by increased serum levels of aspartate aminotransferase (AST) and alanine aminotransferase (ALT) (287). Seropositivity to *C. pneumoniae* was significantly higher in men with non-alcoholic steatohepatitis (288). In rats *E. coli* LPS and protease administration into gingival sulcus resulted in steatosis and hepatic inflammation (289). Also ligature-induced

periodontitis in rats induced hepatocytic lipid droplet formation (290). Thus these non-hepatic infections may play a crucial role also in the liver function.

1.3.3. Steatosis and atherosclerosis

Non-alcoholic fatty liver disease is associated with many features of metabolic syndrome (MetS), such as abdominal obesity, insulin resistance, dyslipidaemia, hypertension, and type 2 diabetes, and can therefore be considered as the hepatic manifestation of MetS (291). Insulin resistance, irrespective of obesity, associates with NAFLD, and is also a risk factor for CVD (292). Epidemiological studies have postulated a link between NAFLD and other non-classical CVD risk factors. These include decreased plasma/serum adiponectin concentrations (293-295), endothelial dysfunction (296), high plasma markers of lipid peroxidation (297,298), and inflammation (299,300). Also the hepatic synthesis of apoB-100, the rate-limiting step of VLDL production and export, is reduced and may result in the retention of lipids in the hepatocytes. The abnormal assembly of VLDL is an important factor in the development of NAFLD, and leads to increased concentration of triglyceride- and cholesterol-rich remnant particles that are atherogenic. (301) A link between these non-classical risk factors for CVD and NAFLD appears to be independent of insulin resistance or other MetS components.

NAFLD is clearly associated with markers of subclinical atherosclerosis. Patients with NAFLD have significantly greater carotid artery IMT and higher prevalence of carotid atherosclerotic plaques than controls, even as early as in childhood (302-306). Most importantly, the positive correlation of the severity of NAFLD and IMT is independent of classical CVD risk factors, insulin resistance or other MetS components (307). Another marker of subclinical atherosclerosis, endothelial dysfunction, also associates with NAFLD as well as the progression of NAFLD (296). A study on type 2 diabetics show a correlation between elevated liver enzyme levels and impaired endothelial function (308). Several studies have presented the connection between NAFLD and existing or future CVD event. The total mortality as well as the mortality to CVD-related incidences is higher in the NAFLD patients than in the general population. (309-311)

Even though the exact biological mechanisms, how NAFLD possesses a risk for CVD, are not yet fully understood, some common factors for these two diseases have been proposed. Such are oxidative stress, decreased levels of adiponectin, insulin resistance, increased levels of CRP, and inflammation. Abdominal visceral fat may secrete proinflammatory cytokines, a risk factor for both NAFLD and CVD. (311) Macrophages of adipose tissue may be a source of cytokines that induce the hepatic expression of SAA, or adipocytes may secrete SAA directly (312-314). Altogether the above mentioned data suggest a clear association between NAFLD and CVD, or that NAFLD may be a risk factor for early development of atherosclerosis.

1.4. Matrix metalloproteinases

1.4.1. Introduction

Matrix metalloproteinases are zinc-dependent enzymes that degrade all components of ECM and basement membrane (BM). MMPs belong to endopeptidases as they cleave the internal peptide bond, and thus far, over 20 MMPs have been identified. The degradation of ECM is essential in normal physiology, such as embryonic development, wound healing, and cell migration (315). However, MMPs are also expressed in several pathological conditions, e.g. in tumour metastasis (316), CVD (317), periodontitis (318) and rheumatoid arthritis (319). MMPs are crucial molecules of inflammatory cells, as they can remodel the ECM thereby facilitating the leukocyte traffic through tissues, such as basement membrane during inflammation (320). Several studies imply that MMPs participate in the rupture of atherosclerotic plaque thereby promoting formation of acute coronary syndrome (ACS).

There are six groups of MMPs: collagenases, gelatinases, stromelysins, matrilysins, membrane-type matrix metalloproteinases (MT-MMPs), and other MMPs, and they exist as two types, secretory MMPs and membrane-bound MMPs. Collagenases, such as MMP-1 and MMP-8, initiate the cleavage of triple helical collagens I, II, and III (321). Gelatinases, like MMP-2 and MMP-9, cleave e.g. native type IV, V, VII, and X collagens and elastin, as well as the proteolytic products of collagens deriving from types I, II, and III after the initial cleavage by collagenases (322).

MMP activity is regulated at the transcription, posttranslational activation, and interaction with inhibitors, the level of transcription being the main level of regulation. Activation of transcription is stimulated by inflammatory cytokines such as TNF- α and IL-1 β (47,323), hormones (324), and growth factors (325). The upregulation MMP-9 can be induced by *C. pneumoniae* HSP60 (326), and by ox-LDL (327) whereas e.g. MMP-8 is inducible by CD40L (41).

Most MMPs are synthesized and secreted as inactive pro-enzymes, zymogens, and the majority of MMPs have a pro-peptide that includes a highly conserved cysteine-containing sequence, so called cysteine switch. This switch binds zinc in the catalytic domain thereby

keeping the enzyme inactive until the covalent bond between cysteine and Zn^{2+} is disrupted by proteolytic cleavage of the pro-peptide domain exposing the catalytic site. (328) Both MMP-8 and -9 are stored as zymogens in secretory granules of neutrophils and eosinophils (329). The activation of zymogens occurs either intracellularly, at the cell surface by MT-MMPs, in the extracellular space by other proteases, or by activated MMPs (stepwise activation). The last holds for the activation of MMP-8 and -9, as e.g. MMP-3 is capable of activating both (330) whereas MMP-9 can also be activated by MMP-2, -7, and -13 (331,332).

In atherosclerotic lesions, MMP activity may result in the migration of vascular SMCs through the internal elastic lamina into the intimal space, where they proliferate thus contributing to the plaque formation (333). The fibrous cap of the atherosclerotic plaque is rich in collagen, especially types I and III, and the breakdown of the cap results in intraluminal thrombosis. MMPs secreted by macrophages are capable of degrading the cap collagen (334). MMPs localize especially to the shoulder regions of the plaque (47,335), the most vulnerable area of the cap. Increased MMP production by foam cells correlates with a thinned fibrous cap (47,336).

The activity of MMPs is inhibited endogenously by specific tissue inhibitors of metalloproteinases (TIMPs) and by non-specific inhibitors, such as α 2-macroglobulin, a serum protease inhibitor that is abundant in the extracellular space and may therefore have an important role in the overall MMP activity in tissues (337). TIMPs inhibit the activity of MMPs by binding to the zinc-binding site of active MMPs at molar equivalence (338). To date, four members of the TIMP gene family have been identified, TIMP-1, -2, -3, and -4, that have 30-40% amino acid identity. TIMP-1, -2, and -4 are secreted in soluble form, whereas TIMP-3 is associated with ECM. TIMP-1 and -2 are capable of inhibiting the activity of most MMPs, and they can form complexes with inactive MMPs. TIMPs are produced by many cell types, such as fibroblasts and endothelial cells. The expression of e.g. TIMP-1 is regulated at the level of transcription by growth factors, cytokines and hormones. TIMPs are not only inhibitors of MMPs but they also have other functions in cell morphology, stimulation of cell growth of different cell types, steroidogenesis, and germ cell development. (339)

1.4.2. MMP-8 and -9 in atherosclerosis

1.4.2.1. MMP-8

Several studies indicate a role of MMP-8, also known as collagenase-2 or neutrophil collagenase, in atherosclerosis. In human plaques, MMP-8 protein and mRNA co-localize with macrophages (340), and their aortic concentration is elevated in patients with abdominal aorta aneurysm (AAA) (341) and ruptured AAA (342). Increased MMP-8 may reflect the state of the disease as its activity is higher in patients with plaque progression (343), and plaques prone to rupture contain more immunoreactive MMP-8 than stable lesions (335). Also a few studies using serum or plasma samples give similar indications; in patients the presence and severity of CAD (344), as well as carotid artery plaque progression (343) are related to elevated MMP-8 concentrations. The concentration of MMP-8 has been reported to be decreased in acute disease progression/end state such as in patients with heart failure (345) or cerebral ischemia (346). MMP-8 pro-enzyme is stored in granules of mature neutrophils - the cells that are first to arrive to the inflammatory site (347). Therefore MMP-8 is released at the initial stage of inflammation and may thus participate also in atherogenesis.

1.4.2.2. MMP-9

Compared to MMP-8, more research has been focused on the role of MMP-9 in CVD. MMP-9 (92-kDa gelatinase or gelatinase-B) is essential for SMC migration (348,349). The cells of the inflamed atherosclerotic plaque, such as macrophages, SMCs, and endothelial cells, are able to produce MMP-9 (45,350). It has been shown both in animals and humans that lipid lowering may reduce expression and activity of MMP-9 likely due to reduced macrophage accumulation (351-353). High serum or plasma MMP-9 concentrations have been reported in patients with unstable angina, AMI (354,355), a history of MI (356,357), unstable coronary plaques (358), and congestive heart failure (345). MMP-9 is highly expressed in the vulnerable regions of atherosclerotic plaques (45), in unstable carotid (359), and coronary plaques (358), in unstable angina (350), in AAA (360) as well as in ruptured AAA (342). In apoE^{-/-}-mice, overexpression of active MMP-9 in the macrophages of advanced atherosclerotic lesions causes plaque disruption (361). In a hypertensive rat model, MMP-9 also promotes the progression of cerebral aneurysm (362). Crossing apoE^{-/-} mice with MMP-9 deficient mice

results in reduced number of lesions and lipid accumulation in aorta as well as decreased lesion macrophage content and media destruction (363). A prognostic significance of plasma MMP-9 was shown by Blankenberg et al., as high baseline MMP-9 level in CAD patients predicted cardiovascular mortality in a four-year follow-up (364). In patients with $\geq 50\%$ carotid stenosis, increased plasma MMP-9 associated with a 2-fold risk for cardiovascular death in a follow up of over four years (365). These factors clearly indicate the important role of MMP-9 in the progression of CVD.

1.4.3. TIMP-1 in atherosclerosis

The balance between MMPs and TIMPs is believed to be crucial in the development and progression of atherosclerosis as it can e.g. block SMC migration by inhibiting MMP-activity (334,366,367). In humans, increased TIMP-1 concentrations or expression have been observed in several forms of CVD. For example patients with ACS and MI have high plasma TIMP-1 concentration, it is upregulated in AAA, and serum TIMP-1 associates with the presence of carotid lesions (355,360,368,369). The predictive value of TIMP-1 for cardiovascular death has been shown in patients with suspected CAD with a follow-up of 2.6 ± 1.2 years (370). Several animal studies have shown the importance of TIMP-1 in the prevention of the atherosclerotic lesion development. In apoE^{-/-} mice, over-expression of TIMP-1 reduces lesions (371). In apoE/TIMP-1-double-knockout mice again, the plaque size reduced and the aneurysm formation, MMP activity, as well as macrophage lipid accumulation increased in the lesions (372). However, study from Lemaitre et al. showed no difference in the lesion size or content of collagen and macrophages in the lesions of the apoE^{-/-}/TIMP-1^{-/-} mice compared to the apoE^{-/-} mice, but the mice exhibited increased pseudo-microaneurysms together with high macrophage content in the media (373). Lipid lowering therapy increases TIMP-1 in carotid plaques further strengthening the conception of a protective role of TIMP-1 in CVD (353).

2. AIMS OF THE STUDY

1. To investigate the effect of *A. actinomycetemcomitans* and *C. pneumoniae* infections on atherosclerotic parameters.
2. To investigate the effect of *A. actinomycetemcomitans* and *C. pneumoniae* infections on the liver.
3. To evaluate the association of serum MMP-8 and TIMP-1 concentrations and CVD events in a population-based cohort with a follow-up time of 10 years.

3. MATERIALS AND METHODS

Materials

Animal studies (I, II, III)

Male apoE^{-/-} mice (Charles River Laboratories, Belgium) were fed a regular mouse chow and kept in germ-free environment. The experiments were conducted in conformity with the Finnish regulations, and the protocols were approved by The Animal Care and Use Committee of the National Public Health Institute, Helsinki, Finland.

Human subjects (IV)

Altogether 2682 Finnish men were enrolled in the Kuopio Ischemic Heart Disease Risk Factor (KIHD) study between 1984 and 1989, and data on their socioeconomic status were collected at the time of sampling. The study was approved by the Research Ethics Committee of the University of Kuopio, Kuopio, Finland, and all subjects gave their written informed consent.

Methods

Method	Publication
Culture and administration of <i>Actinobacillus actinomycetemcomitans</i>	I, II, III
Culture and administration of <i>Chlamydia pneumoniae</i>	II, III
<i>En face</i> morphometric analyses of aortas, Sudan IV staining of lipids	I
Aortic cryosections	I, II
Oil Red O lipid staining of cryosections (aorta, liver)	I, II, III
MMP-8 staining of aortic cryosections	I
MMP-9 staining of aortic cryosections	I, II
CD68 staining of aortic cryosections	I
Serum cholesterol concentration	I, II, III, IV
Serum triglycerides concentration	I, II, III, IV
Serum apoA-I concentration	I, II, III
Serum PLTP activity	I, II, III
Serum LPS activity	I, II, III
Serum A. actinomycetemcomitans IgA and/or IgG concentrations (ELISA)	I, III, IV
Mouse CRP concentration	I
Serum LBP concentration	I, III
Mouse pro-MMP-9 concentration	I
Zymography of serum MMP-9	I

Serum lipoprotein profiles	I
Lipoprotein isolation	I
Phospholipid concentration of lipoprotein fractions	I
Total protein concentration of lipoprotein fractions	I
SAA concentration	II, III
TNF- α concentration	II
sICAM concentration	II
sVCAM concentration	II
sE-selectin concentration	II
MPO staining of aortic cryosections	II
Acetylation of LDL	II
[3 H]-cholesteryl oleate labelling of ac-LDL	II
Mouse peritoneal macrophage cholesterol uptake	II
Mouse peritoneal macrophage cholesterol efflux	II
HE-staining of paraffin sections	III
Steatosis and inflammation grading of liver paraffin sections	III
Liver lipid (PL, cholesterol, TG) analyses	III
Liver fatty acid analysis	III
Tissue RNA isolation	III
cDNA synthetization	III
qPCR	III
Serum ALT concentration	III
Serum adiponectin concentration	III
Serum non-esterified fatty acid concentration	III
Serum phospholipid concentration	III
Endothelial function measurement	II
Plasma fibrinogen concentration	IV
Serum MMP-8 concentration (IFMA)	IV
Serum TIMP-1 concentration (ELISA)	IV
Statistical analyses	I, II, III, IV

Study design (I, II, III)

In publication I, 10 mice were divided into three groups, 3-4 mice/group, and were given weekly *A. actinomycetemcomitans* injections for 4, 6, or 8 weeks. Corresponding control groups received vehicle. All mice were killed one week after their last injection.

For publications II and III, a total 59 mice were used. For the short, 14-week experiment, 20 mice were divided into three groups. Group one was inoculated with *C. pneumoniae* strain Kajaani 7 once (acute infection), group two received three inoculations (chronic infection), and the control group received vehicle. In the longer, 24-week experiment, 39 mice formed four groups, of which group one received weekly *A. actinomycetemcomitans* injections, group two was inoculated intranasally three times with *C. pneumoniae*, group three received both

infections, and group four received vehicle. The intervals of inoculations are depicted in the table below.

Cholesterol uptake and efflux experiments (II)

The efflux capacity of peritoneal macrophages of the mice was measured. Macrophages from each mouse were cultured overnight and the conditioned media were collected after which they were cultured with [³H]-cholesteryl oleate -labelled acetylated LDL (ac-LDL) for 24 hours. After equilibrating the cells overnight in fresh media, the cells were incubated with (ABCA1-mediated efflux) and without (spontaneous efflux) human apoA-I for 5 hours. Radioactivity was measured from the media and from the cell lysates. The efflux was calculated as a percentage of the activity in the medium from the total activity found in the well per protein (µg/ml). The uptake was measured directly from the cell lysates.

The ability of the sera of the infected mice to function as cholesterol acceptors was measured by loading RAW264.7 cells with ac-LDL as previously described, followed by a 5-hour incubation with or without 2% serum isolated from each mouse. The acceptance of free cholesterol by the mouse sera was measured by labelling RAW264.7 cells with [³H]-cholesterol with the same incubation time and serum concentration as above.

Endothelial function test (II)

We tested how macrophages of infected animals affect blood vessels systemically. We isolated peritoneal macrophages from each mouse and cultured them overnight. Conditioned media were collected and a 500 µl pool of each study group was formed. Rat superior mesenteric artery pieces were incubated in these pools in +37°C for 30 minutes, and approximately 5 mm long pieces (6-8 pieces/group) were placed in organ bath chambers. The function of endothelium was determined by precontraction with phenylephrine followed by acetyl choline induced relaxation. The actual experiments were performed twice by contracting the vessels with phenylephrine (1×10^{-6} mmol/l) and thereafter inducing cumulative relaxation with acetylcholine (1×10^{-10} – 1×10^{-5} mmol/l). Finally, the vessels were contracted with phenylephrine followed by 10-minute HDL-induced (10 µg/ml) relaxation.

Table. Timetable of the mouse studies in publications II and III.

		Age (weeks)	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	
Group	14 week	Acute Cpn					□	†											
		Chronic Cpn	□		□		□	†											
		Control	■		■		■	†											
	24 week	A.a.						○	○	○	○	○	○	○	○	○	○	○	†
		Cpn	□		□		□												†
		A.a.+Cpn	□		□		□	○	○	○	○	○	○	○	○	○	○	○	†
		Control (vehicle)	■		■		■	●	●	●	●	●	●	●	●	●	●	●	†

Cpn, *Chlamydia pneumoniae*

Aa, *Aggregatibacter actinomycetemcomitans*

□, *C. pneumoniae* inoculation

○, *A. actinomycetemcomitans* inoculation

■/●, Vehicle inoculation

†, sacrifice

4. RESULTS AND DISCUSSION

4.1. ApoE^{-/-} mouse studies; the effect of *A. actinomycetemcomitans* and *C. pneumoniae* on atherosclerosis-related parameters and the liver

In the original publications I, II, and III, mice were given *A. actinomycetemcomitans* and/or *C. pneumoniae*, and their effects on various parameters associated with atherogenesis and liver characteristics were determined. Hearts, aortas, livers, lungs, sera, and peritoneal macrophages were collected.

4.1.1. *A. actinomycetemcomitans* -induced changes in atherosclerosis-related parameters

Inflammatory markers (I, II)

In publications I and II, several atherosclerosis-associated inflammatory markers were measured. Of these, serum LPS activity (II) as well as CRP (I), SAA (II), and TNF- α (II) concentrations were clearly elevated in the infected mice compared to the controls. In publication I, serum LPS activity had an increased trend in the infected mice. The histochemistry of aortic sinus showed an increased trend of CD68 (I) and neutral lipid content (I) whereas MMP-9 was either significantly (I) or non-significantly (II) increased in the aortic sinuses of the infected mice compared to the controls. This is in accordance with the overall serum MMP-9 activity, as measured with zymography (I), which was higher in the infected groups.

Narrowing of arteries is not a necessary marker of forthcoming complications, since half of the infarctions occur in arteries with <50% stenosis. Therefore, the treatment should not solely focus on this factor, but also on inflammation, which is the main feature of ruptured plaque. The basic methods for combating CVD risk factors, such as exercise, low-cholesterol diet, smoking cessation, and statins, all have also effects on the total inflammatory status of the body (374). Inflammatory markers, such as CRP, SAA, fibrinogen, and TNF- α , have become more and more used when predicting possible CVD risks and events. Our results show that i.v. -administered *A. actinomycetemcomitans* mimicking the bacteraemia in periodontitis, can induce expression of atherosclerosis-related inflammatory markers indicating the possible proatherogenic effect of periodontal infection. The observed increase

in MMP-9 is in accordance with earlier studies showing its role in the development of atherosclerosis in apoE^{-/-} mice (363,375) as well as CVD in humans (350,355,358,359,376). MMP-9 localizes in the shoulder regions of atherosclerotic plaques, the sites prone to rupture, where also inflammatory cells, such as macrophages, accumulate. Activated macrophages are able to secrete MMP-9 (350), and the secretion can be induced by TNF- α and LPS (46,377). The increase in TNF- α and LPS concentrations in our study may have further enhanced MMP-9 production. Another inducer of MMP-9 production in monocytes is modified LDL such as enzymatically degraded LDL and ox-LDL (327,378). In publication I, LDL modification was detected (see “Lipoprotein analyses and aortic lipid accumulation”). The proatherogenic properties of MMP-9 also include potential of processing proinflammatory IL-1 β from its precursor (379) and induction of vascular SMC proliferation and migration (333).

Our results are in accordance with earlier studies on another periodontal pathogen, *P. gingivalis*, showing that oral infection of apoE^{-/-} and intravenous injections of apoE^{+/-} mice accelerated atherogenesis and induced host response (138,139,380). The increased SAA concentration indicates proatherogenic changes, as in addition to its HDL modifying properties, it has been suggested to induce retention of HDL in the arterial wall via binding to proteoglycans (381). All these results indicate an association between *A. actinomycetemcomitans* -infection and inflammatory changes in apoE^{-/-} mouse model. These data indicate that the direct access of periodontal pathogens to the systemic circulation increases atherosclerosis-related inflammatory markers and may be regarded as one of the infections associated with CVD development.

Lipoprotein analyses and aortic lipid accumulation (I)

In publication I a significant increase in serum cholesterol and TG concentrations were seen in the experimental group, which received 8 injections of *A. actinomycetemcomitans*. In addition, the lipoprotein profile demonstrated a shift toward smaller VLDL+IDL and HDL particle sizes with increased cholesterol concentrations. However, *en face* analysis of the whole aorta did not show clear difference in lipid accumulation between the groups. The infected mice showed increased protein and LPS content in VLDL and LDL fractions. The increased protein content reflects a possible decreased particle size; indeed, this was detected in the lipoprotein profile analyses. As all lipoprotein classes, especially HDL, participate in the clearance of LPS, and as in this study there were no differences in LPS contents of the HDL fractions, it can be assumed that the excess LPS administered was addressed to VLDL

and LDL fractions. No differences were observed in cholesterol and TG concentrations in the experiments of publication II, which may be due to the different study design.

The alterations seen in study I have been shown in humans during acute phase response (244). The change in HDL subclass distribution may be a result of e.g. slightly increased PLTP activity which was observed in study I. (247,382) *A. actinomycetemcomitans* infection appears to result in proatherogenic lipid composition, namely decreased VLDL and LDL sizes. (111,383) Also the change in HDL size may affect atherosclerosis formation as even though small HDL particles can function as cholesterol acceptors in reverse cholesterol transport, larger HDL particles have higher efflux capacity and thus are considered more anti-atherogenic (384).

Endothelial function (II)

We isolated and cultured peritoneal macrophages of each infected mouse. For endothelial function tests, conditioned medium pools of cultured peritoneal macrophages from each infected mouse group and controls were formed. We mimicked a vessel response to activated macrophages by incubating pieces of rat superior mesenteric arteries in these medium pools. The endothelial function of the vessels was determined thereafter in an organ bath chamber. The ability of HDL was significantly better to relax the vessel incubated in the pool of conditioned media of the *A. actinomycetemcomitans* group compared to the medium pool of the control group indicating improved endothelial function. Interestingly, the relaxation curve of the infected group vs. the control group showed enhanced relaxation trend also with acetyl choline. In addition, at the acetyl choline concentrations of 3.3×10^{-9} and 1×10^{-8} mmol/l the difference in relaxation was significant.

To our knowledge, there are no previous studies demonstrating how directly administrated *A. actinomycetemcomitans* or *A. actinomycetemcomitans* activated macrophages affect endothelial function. From periodontal pathogens, oral challenge of *P. gingivalis* increases aortic VCAM-1 expression in apoE^{-/-} mice (139) indicating that oral bacteria can affect endothelial function. This may happen by e.g. invading endothelial cells as has been shown with *P. gingivalis* (66). Periodontitis patients show impaired endothelial function compared to healthy controls, and treatment of periodontitis improves the function (104,270,385-387). However, in our study, no significant changes were observed in serum sVCAM-1, sICAM-1, or sE-selectin in the *A. actinomycetemcomitans* -infected mice, and the relaxation studies

showed improved endothelial function, which is contradictory to earlier studies and further studies are needed to verify the results.

Cholesterol uptake and efflux studies (II)

The cholesterol uptake and efflux of peritoneal macrophages as well as the efflux capacity of sera isolated from infected mice were measured. The cholesterol uptake by the peritoneal macrophages of the *A. actinomycetemcomitans* mice was significantly elevated compared to the control group. The ABCA1-mediated efflux was non-significantly impaired, whereas no differences were seen in spontaneous efflux. The efflux ability of sera of the infected mice showed no remarkable differences compared to the controls.

The expression of SR-A is increased in infections (77), thereby increasing the uptake of modified LDL. ABCA1 expression is decreased upon macrophage stimulation with LPS or e.g. TNF- α thereby reducing the efflux (75). As in our study both LPS and TNF- α concentrations were significantly increased in the *A. actinomycetemcomitans* -infected mice, the observed trend to reduced efflux from the peritoneal macrophages may well be attributable to these factors, whereas the increased uptake most likely is a result of increased SR-A expression. Unfortunately, we did not have the possibility to determine the macrophage ribonucleic acid (RNA) expression levels of these receptors.

4.1.2. *C. pneumoniae* -induced changes in atherosclerosis-related parameters

Inflammatory markers (II)

Chronic *C. pneumoniae* infection of the 14-week study increased serum LPS activity (ns.) and SAA concentration which was significantly elevated in the group with acute infection. Immunohistochemical staining of the aortic sinus showed increased MMP-9 expression in the acute group.

The data show that acute *C. pneumoniae* infection results in acute phase response depicted by elevated serum SAA levels and a slight increase in LPS activity. This is in accordance with earlier studies, since even though the biological activity of cLPS is lower than that of enterobacterial LPS, it still induces cytokine production and immune responses (388). The elevation of MMP-expression by *C. pneumoniae* has also been shown in *in vitro* studies with

mouse peritoneal macrophages (326). This supports a study in which *C. pneumoniae* -infection accelerated atherosclerosis progression in apoE^{-/-} mice (159). In a LDLr^{-/-}/apoE^{-/-} double knock-out mouse model, *C. pneumoniae* increased MMP-9 immunoreactivity in aortic arch (389), which is in accordance to our results of the aortic sinus. Like in *A. actinomycetemcomitans* -infected mice, *C. pneumoniae* induces expression of acute phase markers that have been shown to be linked to atherosclerosis.

Lipoprotein analyses (II)

Acute infection with *C. pneumoniae* increased serum PLTP activity, but no differences were seen in serum cholesterol or TG concentrations.

Infections cause increased TG concentrations in humans (390) but again no differences were observed in *C. pneumoniae* -infected mice in our study. The fact that we did not detect changes in cholesterol concentrations is similar to the results of an earlier study on apoE^{-/-} mice with chronic *C. pneumoniae* infection (159). Similarly, a six-month study with *C. pneumoniae* inoculation twice a week resulted in a significant increase in atherosclerotic lesion areas in LDLr^{-/-} mice fed a high cholesterol diet, but no differences were detected in total cholesterol concentrations (161). The ability of *C. pneumoniae* to induce atherosclerosis seems to require high serum cholesterol concentration (162,164), which is naturally more elevated in apoE^{-/-} mice than in LDLr^{-/-} mice on normal chow diet. Without hyperlipidemia, *C. pneumoniae* causes only an inflammatory response (167). The acute infection increased PLTP activity similarly as was observed in study I in the mice infected with *A. actinomycetemcomitans*. Therefore it seems likely that in these mice, PLTP activity is increased due to acute phase response as has also been observed in humans (247,391), but normalizes when the infection has become chronic. In NIH/S mice again, acute *C. pneumoniae* infection did not result in significant changes in the serum PLTP activity (392).

Endothelial function (II)

In the 14-week study, both sICAM and sVCAM concentrations were significantly higher in the chronic as well as in the acute infections, and the HDL-mediated relaxation of the vessels was clearly impaired in these groups. According to Tukey's Multiple Comparison Test, incubation in the macrophage medium of the chronic infection group (14-week study) significantly decreased the ability of acetyl choline to relax the incubated vessel. The difference was significant at the acetyl choline concentration of 3.3×10^{-8} mmol/l. In the 24-

week study, a similar trend as in *A. actinomycetemcomitans* group was observed in the acetyl choline induced relaxation curve. Also HDL-induced relaxation showed improved endothelial function in this *C. pneumoniae* group of the 24 week study.

C. pneumoniae has been shown to impair endothelial function possibly by hampering endothelial NO production. It also induces VCAM-1 expression in apoE^{-/-} mice (393,394) most likely due to the ability of the pathogen to invade and multiply in endothelial cells (79,170). Relevant to our experiment are the results showing that *C. pneumoniae* is able to infect and systemically spread via macrophages (395) thus likely inducing expression of inflammatory mediators that may affect the endothelial function. As in our 14-week study HDL had impaired ability to relax the vessels and the relaxation capability of acetyl choline was reduced, the results indicate a direct role of this infection in NO availability and endothelial dysfunction. However, the impact of acute infection seems to be more profound as there were no similar changes in the chronic *C. pneumoniae* group of the 24-week study. This is further supported by a study conducted in pigs showing that acute *C. pneumoniae* infection causes endothelial dysfunction (396). The induced production of VCAM-1 in the *C. pneumoniae* -infected mice compared to E-selectin in animals with dual infection (*A. actinomycetemcomitans* and *C. pneumoniae*) may reflect a slightly different response: E-selectin preferentially recruits Th1 cells whereas VCAM-1 is rather involved in Th2 cell recruitment (397).

Cholesterol uptake and efflux studies (II)

The cholesterol uptake by the peritoneal macrophages isolated from the *C. pneumoniae* -infected mice showed no differences compared to the controls. Spontaneous efflux was significantly higher in all *C. pneumoniae* -infected groups. An opposite but a non-significant trend was observed in the ABCA1-mediated efflux. The efflux capacity of the sera isolated from these mice was slightly lower (n.s.) in both chronic groups compared to their controls.

The increased spontaneous efflux may be a result of increased diffusion or activity of SR-BI, but their role in our experiments needs further studies. As the sera from the mice with the chronic *C. pneumoniae* could not function properly as cholesterol acceptors, we may hypothesize that in these mice the function of HDL is impaired due to its inflammation-related modifications. As previously demonstrated during infection, HDL concentration decreases, its composition changes, and its efflux capacity decreases (112).

4.1.3. A combined *A. actinomycetemcomitans* and *C. pneumoniae* infection - induced changes in atherosclerosis-related parameters

Inflammatory markers (II)

The dual infection elevated serum LPS activity as well as TNF- α and SAA concentration, but the results did not reach statistical significance. The immunohistological stainings of MMP-9 and MPO in the aortic sinus showed increased immunoreactivity.

As outlined above, dual infection evoked an inflammatory response as demonstrated by the increased concentrations of inflammatory markers compared to mice with single infection. This is the only group with elevated MPO production in the aortic sinus indicating that the infectious burden may impair the function of HDL in cholesterol efflux by increasing MPO, an HDL-oxidizing enzyme (230,398), production in atherosclerosis-prone areas.

Endothelial function (II)

From endothelial function markers, serum sE-selectin concentration was significantly increased in the group of combined infections. In relaxation experiments, the study group had clearly reduced HDL-mediated relaxation of the vessel compared to the control group. A similar effect was observed in acetyl choline induced relaxation curve with a significant difference at the concentrations of 1×10^{-9} , 3.3×10^{-9} , and 1×10^{-8} mmol/l.

The co-infection had an effect on the endothelial function as seen in the increase of serum sE-selectin concentration and reduction of both acetyl choline and HDL-induced relaxations of the rat vessels. These results are in accordance to a study where Liuba et al co-infected apoE^{-/-} mice with *C. pneumoniae* and *Helicobacter pylori* (394). A serological study has also shown the pathogen burden to be an independent predictor of endothelial dysfunction (265). As HDL was not able to induce relaxation of the vessels, this indicates that the factors secreted by inflammatory cells directly affect the endothelium and thus promotes functions related to early atherosclerosis formation, i.e. endothelial dysfunctions. Therefore it is possible that the actual infection site is not necessarily present in the vasculature but affects systemically - as could be the case in periodontitis. Endothelial activation can be induced by e.g. LPS, bacteria, and TNF- α , leading to a release of pro-inflammatory cytokines by endothelial cells. They may also down-regulate SR-BI, which mediates HDL-induced relaxation (399). In diseased endothelium, the synthesis and bioactivity of vasodilators, such as NO, is reduced leading to

redox imbalance. Increased ROS production enhances NO degradation, thus making the endothelium more dysfunctional and leading to increased adhesion of inflammatory cells and SMC proliferation (253). There are several studies that have assessed the relationship between endothelial function and inflammation markers such as CRP, sVCAM-1, and sICAM-1, showing mainly inverse correlation (reviewed in (266)). Our results indicate that the dual infection with *A. actinomycetemcomitans* and *C. pneumoniae* induces endothelial dysfunction, probably also via increased TNF- α production promoted by the challenge of LPS.

Cholesterol uptake and efflux studies (II)

The cholesterol uptake by the macrophages of the group with combined infection was slightly elevated. Of efflux studies, spontaneous efflux showed a significant increase compared to the control group. The efflux capacity of the sera in this group, however, was significantly decreased.

This is the only group with a decreased efflux capacity of the sera indicating that the HDL has been modified making it less able to accept cholesterol. The dual infection therefore affects HDL's ability to remove excess cholesterol in reverse cholesterol transport thereby promoting atherosclerosis formation.

4.1.4. *A. actinomycetemcomitans* and *C. pneumoniae* infection -induced changes in the liver

4.1.4.1. The effect of *A. actinomycetemcomitans* on the liver (III)

Administration of *A. actinomycetemcomitans* induced moderate liver inflammation with inflammatory cell infiltration. *A. actinomycetemcomitans* was detected by PCR in the livers of 30% of the mice infected with *A. actinomycetemcomitans*. Liver phospholipid concentration was decreased, which was also reflected in the increased liver TG/PL ratio. These changes may result from increased liver phospholipase A1 and A2 activities, which are induced by LPS (400). No changes were detected in liver TG or cholesterol concentrations, nor in unsaturated or saturated fatty acid proportions compared to the controls. Only anti-inflammatory dihomo γ -linoleic acid (DGLA) showed a relative increase in the infected group. DGLA may be considered anti-inflammatory as it decreases the pro-inflammatory effects of arachidonic acid (ARA) (401). Expression studies showed elevated expression of

inflammatory markers IL-1 β and CD68, a macrophage marker, as well as MCP-1 and FAS. Periodontitis has been linked to liver inflammation in animal models (290), which is in accordance to our results. Altogether the association between periodontitis and liver alterations has been scarcely studied. Our results indicate that constant exposure to *A. actinomycetemcomitans* causes inflammatory response in the liver as demonstrated by histology and expression studies.

4.1.4.2. The effect of *C. pneumoniae* on the liver (III)

C. pneumoniae was detected in the lungs (100% and 55%) and livers (21% and 0%) of the infected mice of the 14-week and 24-week studies, respectively. Chronic infection resulted in hepatic microvesicular appearance (14-week and 24-week study) and promoted liver inflammation (24-week study). Liver phospholipid concentrations were significantly decreased in all *C. pneumoniae* infected groups, but no changes were detected in liver TG and cholesterol concentrations. The only exception was the decreased TG concentration in the acute group compared to the control. The livers in the acute group were also depleted of lipids as was shown with lipid staining. The TG/PL ratio was lower in the acute *C. pneumoniae* group and higher in both chronic *C. pneumoniae* groups compared to the controls.

Hepatic saturated fatty acid content was clearly increased in acute infection, but decreased in chronic infections, especially in the 24-week study. Of unsaturated fatty acids, acute infection resulted in reduced MUFA content, whereas chronic infection increased the proportion of monounsaturated with a concomitant decrease of polyunsaturated fatty acids (PUFA). In the 14-week study, chronic infection increased DGLA proportion, and its precursor, γ -linoleic acid (GLA), was correspondingly decreased in both acute and chronic infections. Acute infection elevated the levels of both anti-inflammatory n-3 PUFA and pro-inflammatory ARA. Arachidonic acid is considered pro-inflammatory since it is a substrate for pro-inflammatory eicosanoids (401). In expression studies, hepatic expression of IL-1 β , CD68, and MCP-1 were induced in acute group, but no differences were observed in chronic groups except for the slight decrease of IL-1 β expression detected in the 14-week study.

In the chronic group of the 14-week study, the observed high proportions of MUFA and distinct hepatic fatty acid profile compared to both the control as well as to the acute infection group indicate an effect of the duration of the infection. The acute group had more

inflammatory changes as was seen in the alterations in the expression studies and e.g. in the proportions of hepatic pro-inflammatory ARA as well as in anti-inflammatory n-3 fatty acids. Altogether, the hepatic fatty acid profile was similar in both chronic groups and differed clearly from that of the acute group.

As has been shown, the liver plays a significant role in the defence against bacterial infection. (402) and e.g. *C. pneumoniae* is able to replicate and induce pro-inflammatory cytokine production in Kupffer cells, the liver macrophages (403). Of the fatty acids, hepatic n-3 PUFAs have a protective role (404), and our study further enhances this view: n-3 PUFA and inflammation correlated negatively with each other. The observed clear reduction in PL concentrations in all infected groups may imply reduction in cell membrane PLs, as seen in mice infected with hepatitis virus (405). Results suggest that the course of *C. pneumoniae* - induced liver response involves first inflammation (acute) followed by a FA imbalance observed in the chronic groups. The histological morphology suggested the presence of mild steatosis, but the lipid staining and expression studies did not support this concept. However, the observed microvesicular appearance and changed fatty acid profile indicate a hepatic response to these infections, but further studies are warranted to specify the findings.

4.1.4.3. The effect of the combined infection with *A. actinomycetemcomitans* and *C. pneumoniae* on the liver (III)

The dual infection resulted in liver inflammation and decreased PL concentration. There were no significant changes in liver TG, cholesterol, SFA, MUFA, or PUFA concentrations compared to controls; only a slight increase in DGLA proportion was detected. Expression of IL-1 β was increased, whereas MCP-1 was clearly downregulated.

The effects of the two infections were not cumulative as contrary to what one could have hypothesized. The minor changes mainly in the inflammatory parameters observed indicate that the first infection with *C. pneumoniae* may have primed the mice and induced an immunological defence. This may have therefore resulted in the marginal differences in parameters observed after the second infection with *A. actinomycetemcomitans*.

4.2. Serum MMP-8, a risk factor for future CVD death

Our study (IV) shows that elevated serum MMP-8 concentration in a 10-year follow-up is an independent risk factor for AMI, CHD, CVD, and all cause death. Especially men with subclinical atherosclerosis (IMT > 1 mm) had a three-fold increased risk for CVD death independently of other risk factors. With increased MMP/TIMP-1 ratio the risk was close to 2.5-fold, but only of borderline significance in the multivariate model. Figure 6 shows the cumulative survival rates for CVD death, with MMP-8, TIMP-1, and MMP-8/TIMP-1 concentrations.

MMP-8 is one of the collagenases that are able to initiate the degradation of triple-helical collagen and therefore it may have a clinical relevance in relation to the weakening of fibrous cap covering the atherosclerotic lesions. As thrombosis occurs in many cases where no substantial occlusions of the vessels appear, a diagnosis of possible weakening or eroding of the fibrous cap by serum MMP-8 could provide useful information when identifying groups in risk and when planning treatments.

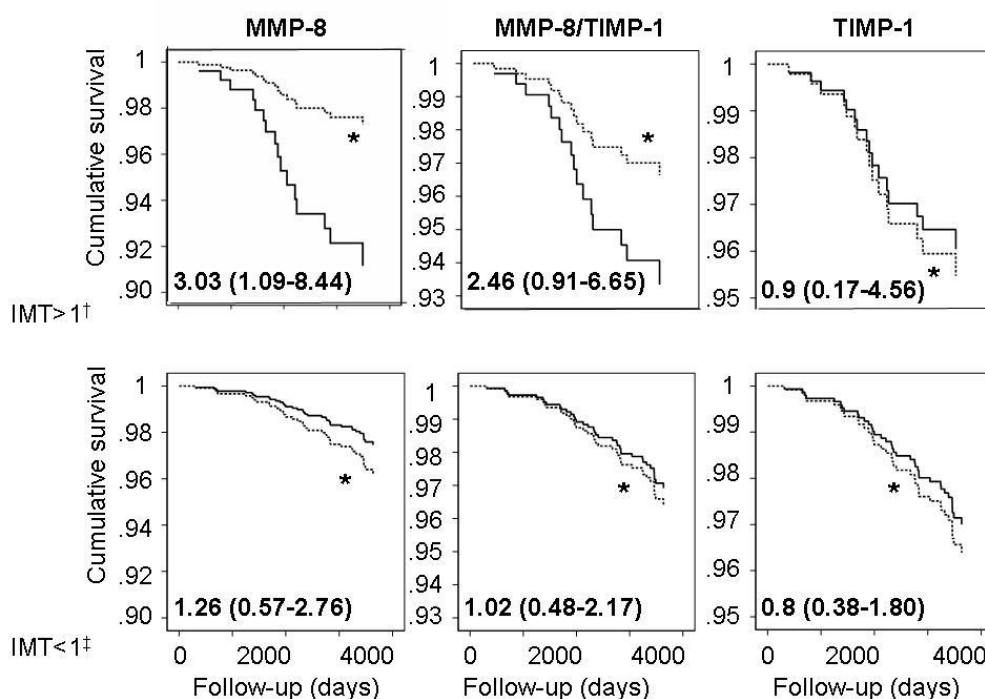


Figure 6. Cumulative survival rates for CVD death with or without increased IMT in the highest quartile (black line) vs. three lower quartiles (dotted line) of measured serum parameters. The relative risks (confidence intervals) are shown in the figure.

† Adjusted for age, body mass index (BMI), smoking, and plasma fibrinogen concentration.

‡ Adjusted for age.

MMP-8 was long thought to be produced only by neutrophils, but it is expressed also in human ECs, SMCs, and macrophages after stimulation with CD40L, TNF- α , or LPS (335). As previously shown, MMP-8 localizes to shoulder regions of advanced atherosclerotic lesions, and it is expressed or elevated in patients with diagnosed CVD or plaque progression, but no prospective studies have been performed before (335,340,343,344). Therefore patients with an indication of subclinical atherosclerosis together with elevated serum MMP-8 concentration may have an ongoing inflammatory process that eventually leads to plaque rupture and acute manifestation of CVD. The concept of inflammatory process behind the elevated MMP-8 concentrations comes from the studies showing that macrophages secrete MMPs (334,406). Also the increased IMT suggests an inflammatory process as the thickening of intima results from e.g. accumulation of lipids and increased SCM content, which both have shown to be present in inflammatory, atherosclerotic lesion formation (1).

A recent study from our laboratory further demonstrates the relationship between MMP-8 and future CVD events (unpublished results). The study comprised of 140 subjects with ACS (acute myocardial infarction or unstable angina pectoris) that were divided into two groups according to the dental status in the panoramic x-rays: edentulous and periodontally healthy patients or patients with periodontitis. The follow-up time for recurrent CVD events (CVD death, MI, unstable angina, or ischemic stroke) was one year, and blood samples were collected at baseline, after 1 week, 3 months, and 1 year. When the whole study population or periodontitis patients were analysed, MMP-8 concentrations were not associated with the recurrent CVD events. However, in the group of edentulous and periodontally healthy patients, serum MMP-8 concentrations were associated with CVD events at baseline and after one week with RRs (95% CI, p) of 2.94 (1.038-8.313, p=0.042,) and 4.89 (1.539-15.540, 0.007), respectively. The results therefore suggest that periodontitis increases MMP-8 concentration and can be considered as a confounding factor. Only in periodontally healthy patients elevated serum MMP-8 concentration was predictive of the risk of CVD event. In our present study (publication IV), the only available information concerning periodontitis, was the serum antibody levels against major periodontal pathogens. No association, however, was found between those and MMP-8 concentration.

According to Herman et al, in acute inflammation, MMP-8 is released from polymorphonuclear granulocytes, but the synthesis and release of MMP-8 from ECs, SMCs, and macrophages seem to require chronic inflammation with a longer exposure to

proinflammatory cytokines (335). These results are in good accordance with our results of MMP-8 being an early player in cap remodelling preceded by an inflammatory condition eventually leading to acute coronary symptoms. Therefore MMP-8 could well serve as a predictive marker of future CVD events.

The importance of the sample type for MMP and TIMP measurements was addressed by Professor Jung (407). His data showed that from the same patients, serum samples had higher MMP-8 and TIMP-1 concentrations compared to plasma samples. Moreover, serum samples collected with a clot activator had higher MMP-8 concentrations compared to serum samples collected without any clot activator. The rationale behind these findings is that MMP-8 and TIMP-1 may be released from platelets and leukocytes during the blood collection or coagulation. In our study, glass tubes were used, as the blood clotting in them is more effective than in plastic tubes and thus no clot activators are needed. We further tested the difference between serum and plasma MMP-8 concentrations with two different methods, namely time-resolved immunofluorometric assay (IFMA) and ELISA (408). With both methods, serum MMP-8 concentrations were significantly higher than those measured from plasma. Notable was that there were significant positive correlations between serum and plasma IFMA as well as serum and plasma ELISA results thereby indicating the suitability of serum for these measurements. In our original publication the samples were collected in a uniform way from each patient and, irrespective of the origin of the MMP-8, the results demonstrate the association between elevated serum MMP-8 concentration and CVD event. Therefore the conclusions can be considered valid.

5. CONCLUSIONS

Our data show that *A. actinomycetemcomitans* and *C. pneumoniae* induce atherosclerosis-related alterations in serum and aorta of apoE^{-/-} mice. Infection with *A. actinomycetemcomitans* induced inflammatory response, increased the amount of MMP-9 in serum and vascular tissue and cholesterol uptake into peritoneal macrophages, and reduced lipoprotein sizes thus transforming them more proatherogenic. Also, *C. pneumoniae* induced increased expression of inflammatory markers as well as impaired endothelial function. The data therefore demonstrate that infections with these pathogens can induce alterations in the vasculature related to early formation of atherosclerosis.

In the liver, *A. actinomycetemcomitans* administration resulted in inflammation. Also *C. pneumoniae* induced inflammation and furthermore microvesicular appearance and fatty acid imbalance. The observed effects show that infections may have a more profound systemic influence than generally recognized. The findings of these alterations indicate that these organisms may adversely influence lipid metabolism, and as a consequence the course of cardiovascular diseases.

In the population-based cohort, men with subclinical atherosclerosis, as reflected by increased IMT, and elevated serum MMP-8 concentration had a three-fold increased risk for CVD death during the 10 year follow-up. The association was independent of other CVD risk factors. As fatal CVD events are in many cases unexpected, our data suggest that serum MMP-8 could be considered as a potential new marker for risk of future CVD death.

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